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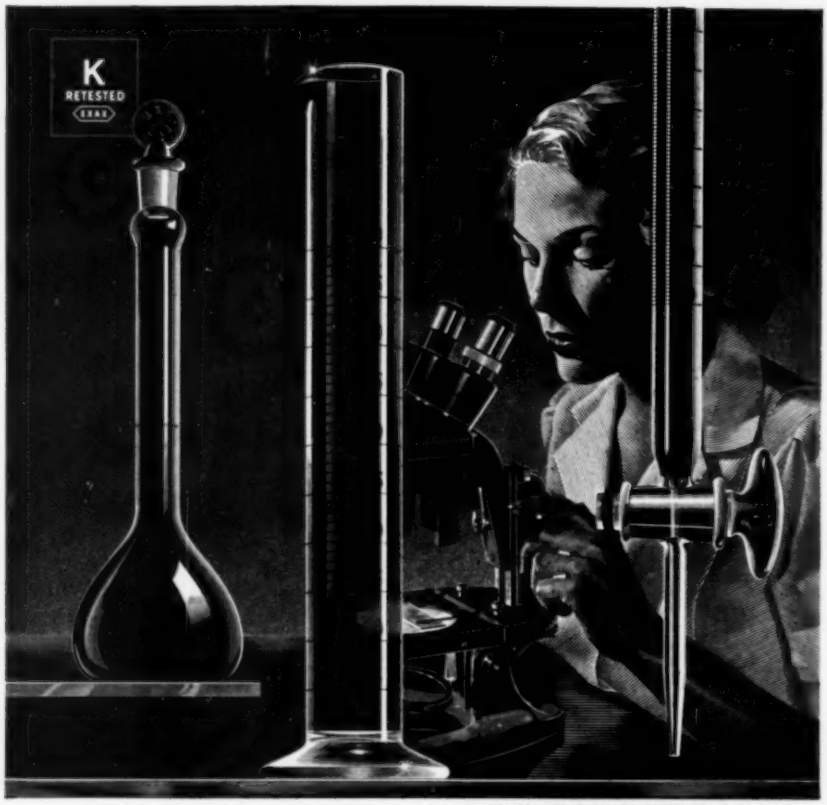

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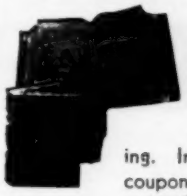

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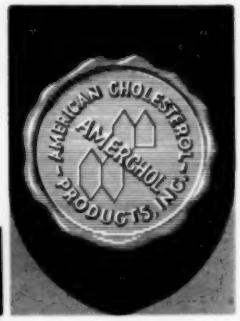

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The Release of Antimicrobial Agents from Solutions of Surface-Active Agents*

By NASEEM A. ALLAWALA and SIDNEY RIEGELMAN†

Surface-active agents modify biological results, but their effect can be related to well-known thermodynamic quantities. New data are presented, and the results of earlier studies are reviewed and explained in terms of the fundamental driving forces behind the availability of antimicrobial agents in such systems.

THE present report is the first of a series relating to the release of drugs from systems containing surface-active agents. A study of these systems shows that the release of drugs from their vehicles is governed by well-known physical laws and can be predicted. The literature on this subject is somewhat controversial because of the confusion resulting from incorrect analysis and interpretation of data. Due to the increasing use of surface-active agents in pharmaceutical formulations, an evaluation has been made of the factors governing the biological effectiveness of drugs as influenced by the presence of surface-active agents.

The purpose of this report is to point out some of the facts which must be known before any conclusions can be drawn with regard to the influence of surface-active agents upon the release of drugs from their vehicles. The knowledge of these facts is essential not only for the analysis of the biological data but also for the proper formulation of pharmaceutical preparations containing surface-active agents. In this paper, therefore, attention will be restricted to the general concepts included in the following topics: (*a*) concept of availability; (*b*) solubilization phenomenon; (*c*) effect of fractional saturation; (*d*) partition coefficient as a measure of availability; (*e*) effect of increase in drug concentration; and (*f*) unique effects of surface-active agents.

* Received August 22, 1952, from the College of Pharmacy, University of California, Medical Center, San Francisco.
† Assistant Professor of Pharmacy, University of California.

CONCEPTS

Availability.—A useful classification of drugs arose from Ferguson's postulate of chemical potential as an index of the biological toxicity (4), and the types of drugs covered in the present paper are restricted in terms of this postulate. Ferguson and others (4, 5) have shown that for structurally nonspecific agents (in contrast, for instance, to competitive enzyme inhibitors which will be structurally specific) the biological activity is related to the thermodynamic activity of the toxic agent in the external phase. One of the basic requirements for the validity of the Ferguson rule is that the toxic action under study must be independent of time or that it must be an equilibrium type, rather than kinetic. The narcotic action of most agents falls within this category, but there seems to be no useful bactericidal agent which can be assumed to have an equilibrium action (except, possibly, acridines). The present report deals with the common bactericidal agents

which are of the kinetic type; i. e., their toxicity is dependent upon time, among other factors. It is felt that Ferguson's postulate can be extended to this type of agent as well, by defining certain terms such as availability and by making justifiable assumptions. The validity of this extension is borne out by the experimental data to be discussed.

From the qualities assigned to the term "availability" and "release," it becomes apparent that availability is a rate quantity dependent upon a driving force, and it is usually measured in terms of a given response. In a certain case, availability may be expressed in grams per unit time required for an antibacterial agent to traverse a bacterial membrane; when this flow is continued for a sufficient length of time, the bacterium is killed. Thus availability in this case is the same as permeability which is defined as $Q = hS(c' - ac)$[1] [(1), Eq. 1]. Availability may also assume other forms, depending upon the conditions used and the response measured. If the response measured is diffusion, then availability is the same as flux and is easily defined as $Q = DSdc/dx$[2] (Eq. 2). If the response involves transfer across an interface, then the availability is expressed by a complicated function of a partition coefficient (2, 3). Finally, when the action of the drug is on the surface of bacteria, the rate of adsorption is the measure of availability and is expressed as $Q = Kc^{1/n}$,[3] (Eq. 3), assuming the surface available for adsorption remains constant. This is the same as assuming that only a small fraction of the total surface has to be covered for lethal action.

It is obvious that these four forms of availability are not identical and cannot be used interchangeably. The factors which influence one of the forms of availability may not influence the others. However, in all cases the driving force is a common determining factor. The driving force measures how far short of equilibrium the system is; the availability measures the rate at which this equilibrium is approached. The true driving force is the partial molal free energy gradient (chemical potential) and is measured in terms of the thermodynamic activity. In testing of antiseptics, the thermodynamic activity in the surrounding medium can be used directly as the measure of the driving force by simply setting up a common standard state for all systems. In accordance with Ferguson (4), the pure solid or its saturated solution is set as the standard state of reference and the activity is expressed in terms of this standard state. Ferguson and others (4, 5) have shown that, since the chemical potential of the toxic substances must be the same in all phases partaking in the equilibrium, the toxicities of the toxic substances (equilibrium type of action) should be compared not with the values of the toxic concentration in the external phase but with the values of the chemical potentials in these phases. From the definitions of the availability as a rate phenomenon, it will be seen that the same situation should be true of kinetic-type action. An additional assumption, which may be necessary in some cases, is that the thermodynamic activity of the drug inside the bacteria is always zero.

Since the chemical potential is an instantaneous quantity in a system not at equilibrium, its rate of change will depend upon the available amount in solution. This amount, the capacity, is proportional to the difference between the driving forces with respect to a given standard state at zero time and at equilibrium. The importance of both the thermodynamic activity and the capacity as factors influencing the availability of antimicrobial agents from the solutions of the surface-active agents will be demonstrated.

The thermodynamic activity of a drug in a solvent depends upon the nature of the solvent and the solubility of the drug in it. In a simple system such as iodine in water, the changes in the thermodynamic activity of the drug are related directly to the changes in both the concentration of the drug and the per cent saturation of the solution. Therefore, the biological availability of the drug is related to its concentration in solution or the per cent saturation. This is illustrated by the data reported by Alexander and Trim (6) which have been replotted in Fig. 1. From this figure it can be seen that when other factors (time, worm body surface area, etc.) are constant, the uptake by Ascaris worm increases as the thermodynamic activity of hexylresorcinol in solution increases. Hence, for a simple solvent, i. e., one without any surface-active agent, knowledge of the changes in the concentration of the drug gives an index of the changes in its thermodynamic activity or antibacterial activity.

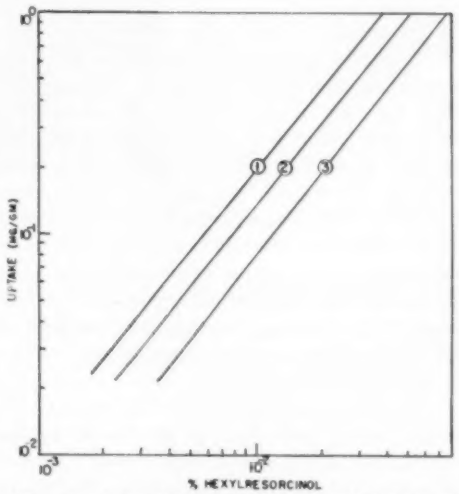

Fig. 1.—A log–log plot of the uptake of hexylresorcinol (mg./Gm. wet weight of ascaris worm) against its concentration in water for any fixed time. 1: forty minutes; 2: twenty minutes; 3: ten minutes. Reconstructed from Fig. 5 in Reference (6).

[1] Equation 1: Q = the permeability or availability in Gm./second; h = the permeability coefficient in cm./second; S = the area in cm.^{2}; c' = concentration outside membrane in Gm./cc.; c = concentration inside membrane in Gm./cc.

[2] Equation 2: Q = the flux or availability in Gm./second; D = the diffusion coefficient in cm.^{2}/second; S = the area in cm.^{2}; dc/dx = the concentration gradient in Gm./cm.^{4}.

[3] Equation 3: Q = the availability in Gm./second; K = a constant in cm.^{2}/second; c = the instantaneous concentration in the surrounding medium in Gm./cc.; n = a pure number.

Solubilization Phenomenon.—There is evidence that the solutions of surface-active agents do not behave as simple solvents. Furthermore, in the presence of the surface-active agent, the concentration of the drug alone is a misleading index of the biological activity of the system.

It has been demonstrated that, unlike simple solvents or miscible solvents, the surface-active agents increase the apparent solubility of the drug above its water solubility by incorporating the drug in micelles, a process known as solubilization (7). These micelles are discrete, self-limiting molecular aggregates which are formed in the bulk of the solution. Although these aggregates are only 50–150 Å. in diameter, they exhibit certain properties of a separate "phase," dispersed as submicroscopic particles (8). The "phase" shows rather high affinity for lipophyllic solutes, competing for the solute with the aqueous environment on a mass law or partition basis. This competition can be illustrated by saturating water with a volatile substance such as ether. This solution will exhibit the partial pressure of ether at that temperature. The addition of the surface-active agent after removal of the undissolved ether phase will depress the escaping tendency or the thermodynamic activity of the ether for below its former value (9). The resultant value will be dependent on the ether freely dispersed in the aqueous phase or environment. In an analogous manner, surface-active agents influence the availability of solubilized drugs.

The maximum amount of the drug which can be solubilized in a solution of the surface-active agent is dependent upon the nature of the surface-active agent, the nature of the drug, the ionic concentration in the medium, the temperature, the presence of nonelectrolytes, and the concentration of the surface-active agent (10). One simple method for expressing solubilization as a function of the concentration of the surface-active agent is illustrated in Fig. 2,[4] which represents the solubility of iodine and hexachlorophene (N. N. R.) in Antarox A-400.[5] This nonionic surface-active agent is an ether formed by the reaction of nonylphenol with approximately 10 moles of ethylene oxide. It was selected for a series of studies to be reported in this paper, since it is extremely stable to acids and alkalis, is free from odor, shows slight batch variations, possesses low toxicity, and lacks antibacterial activity (11–13).

It is important to note the differences between solubilization by surface-active agents and the increase of solubility caused by the addition of a miscible solvent. This is illustrated for iodine in Fig. 3. High concentrations of a miscible solvent like alcohol are required before the solubility of iodine becomes appreciable. In contrast, the solubilization by surface-active agents is significant even in extremely dilute solutions, and it is due to this fact that the surface-active agents offer a definite advantage in formulation.

Effect of Fractional Saturation (14–18).—Knowledge of the mode and the nature of solubilization

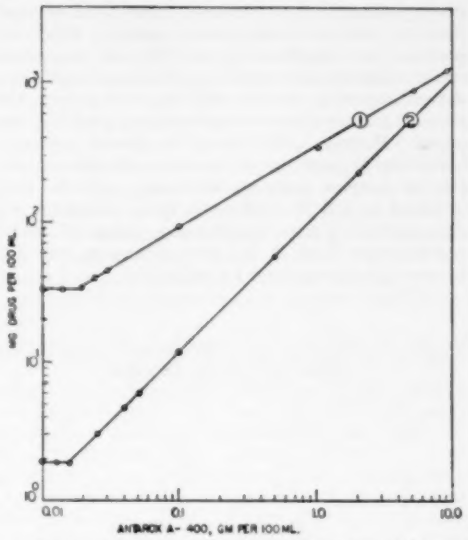

Fig. 2.—A log–log plot of total drug (mg./100 ml.) in solution *vs.* concentration of Antarox A-400 (Gm./100 ml.) at 25°. 1: iodine; 2: hexachlorophene (G-11).

enables one to appreciate the findings of Tilley and Schaeffer (19) and Hampil (20). Their work offers the first example of studies of the effective-

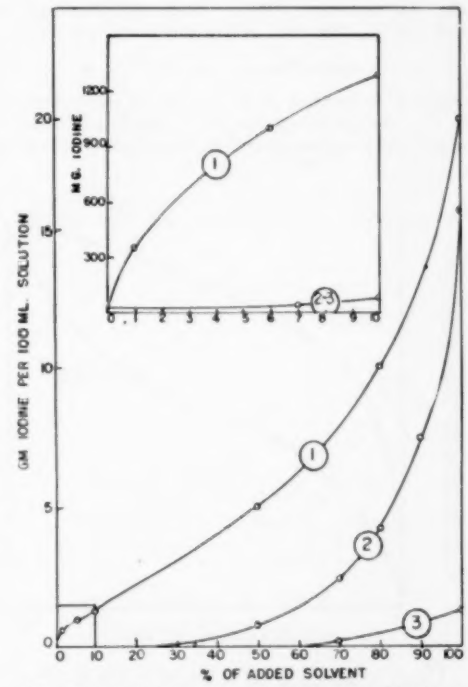

Fig. 3.—A plot showing solubilization of iodine *vs.* solvent action in aqueous solutions containing, 1: Antarox A-400 (% w/v) at 25°; 2: ethyl alcohol (% v/v) at 15°; 3: glycerin (% w/w) at 25°.

[4] Details on the significance of this method of plotting are presented elsewhere.

[5] Antara Products, a division of General Aniline and Film Corporation, New York 22, N. Y.

ness of an antibacterial agent when used in conjunction with a surface-active agent. Tilley reported that antibacterial activity of saponated cresol solutions expressed by the phenol coefficient varies more than several fold, depending upon the amount and the kind of soap used to solubilize the cresol. Hampil (20) followed a similar approach involving preparation of solutions of phenol containing varying amounts of soaps, and the time required to kill *E. typhosa* in these solutions was determined. These observations, some of which are listed in Table I, led her to propose that the phenols are inactivated by soaps.

TABLE I.—ANTIBACTERIAL ACTIVITY OF SAPONATED CRESOL SOLUTIONS (HAMPIL)

Sod. Oleate, %	Phenol, %	Saturation, %	Killing Time, Min.
5	0.6	0.3	>2
2	0.6	1.8	1
5	1	0.5	<2
4	1	0.75	1

Hampil's proposal seems reasonable only if it is assumed that the addition of soap does not change the nature of the solvent or that the thermodynamic activity of phenol in all solutions studied is identical. Actually, both of these assumptions are incorrect. From knowledge of the solubility of phenol as a function of the concentration of sodium oleate (21), the per cent saturation of these solutions can be calculated. Per cent saturation is defined as:

$$\% \text{ saturation} = G \times 100/S - S^{\circ}$$

where G = amount of drug in wt. or moles present in the solution referred to, S = solubility in surface-active agent solution, and S° = solubility in water.

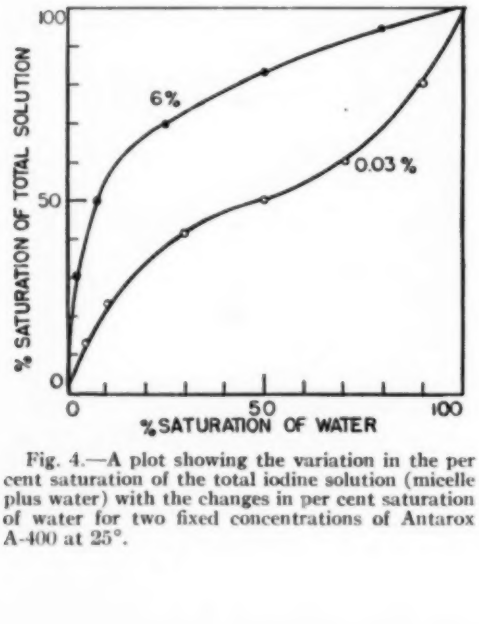

Fig. 4.—A plot showing the variation in the per cent saturation of the total iodine solution (micelle plus water) with the changes in per cent saturation of water for two fixed concentrations of Antarox A-400 at 25°.

In the third column of Table I, per cent saturation of the solutions have been included. It can immediately be seen that the antibacterial activity of the solution falls with the decrease in per cent saturation, even though the total amount of phenol in solution remains constant. Applying the concept already developed, it can be stated that if an estimate of the antibacterial activity is to be made it is essential to know not only the concentration of the antimicrobial agent but also the per cent saturation in the solvent used. Equitoxic solutions of a given toxic material in different solvents would be those in which the thermodynamic activity or the per cent saturation are identical, and not the actual concentrations.

It is reasonable to say that phenol in a surface-active agent solution will be distributed between the micelles and the surrounding water, and that it will be the phenol in water which will have the antibacterial action. Due to the nature of solubilization, the thermodynamic activity of the drug is related to the per cent saturation of the water rather than that of the total solution (micelle and water combined), even though the two are closely related. Therefore, for quantitative correlation the per cent saturation of the water phase should be used as an index of the antibacterial activity. Figure 4[6] shows the per cent saturation of the solution as a function of the per cent saturation of water for iodine solubilized in Antarox A-400. It is to be noted that the per cent saturation of the total solution is greater than the per cent saturation of the water; the effect increases with higher concentrations of the surface-active agent. In other words, the drug is withdrawn into the micelle to a greater extent. Unfortunately no equivalent data are available for the phenol sodium oleate system.

Table II shows the correlation between the biological activity of solubilized iodine and its thermodynamic activity as expressed by the water concentration. Only the absolute amount or the per cent saturation of iodine in the water can be quantitatively correlated with the killing time. Alexander and Tomlinson's data (22) further confirm this concept. They report that in the presence of Aerosol MA, the killing time (bacteriological action on a coliform organism) was inversely related to the free phenol in water over the narrow range of concentrations investigated.

If the per cent saturation were the only factor, all the saturated solutions would have the same biological activity even if the solvent was one in which the drug was insoluble. However, this is not actually the case, since experiments have shown that at 25°, 33 mg. of iodine is required to saturate 100 cc. of water, whereas 1,000 mg. is required to saturate 100 cc. of 6% Antarox Sol. If 30 mg. of iodine was removed from each of these systems, the thermodynamic activity of iodine in water would be lowered tremendously, whereas the change in the other solution would hardly be measurable. Therefore, not only the thermodynamic activity

[6] The iodine data presented in this paper are abstracted from the complete investigation reported in the article by Allawala, N. A., and Riegelman, S., entitled "The Release of Antimicrobial Agents from Solutions." The experimental procedure is given therein.

TABLE II.—CORRELATION BETWEEN BIOLOGICAL AND THERMODYNAMIC ACTIVITY OF SOLUBILIZED IODINE

Concn. Ant. A-400, % w/v	I_2 in Soln., —Mg./100 Cc.— Micell.	Aq.	—% Satn.— Total Soln.	Aq.	99% Killing Time, Min.
10	1230	33[a]	100	100	3.5
0.085	81	33	100	100	3.8
9.3	1000	10	82	33	6
6.5	610	3.6	60	11	9
0.085	15	2	17	6	12

[a] 100 cc. of water dissolves 33 mg. of I_2 at 25°.

must be known but also the capacity of the systems involved. The exact interrelationship between the thermodynamic activity (the intensity factor) and the saturation solubility (the capacity factor) cannot be discussed here, but both are involved among the factors governing the availability of drugs.

Partition Coefficient as in Index of Availability.—Since the capacity or the maximum amount which can be solubilized increases as concentration of the surface-active agent is increased, it is necessary to know how the partition coefficient changes with the increasing concentration of the surface-active agent.

The partition coefficient, it will be recalled, is the ratio between the concentrations of the drug in the two phases at equilibrium. By selection of the pure drug or its saturated solution as a standard state of reference, the existence of an equilibrium defines the thermodynamic activity as equal in these two phases. The partition coefficient is not a rate quantity, but the rate of approach of equilibrium may be related to it (2, 23). The partition coefficient between the vehicle and the body fluids is thus not a measure of the availability, but it is a commonly used index of the usefulness of the vehicle.

Light liquid petrolatum was chosen for a portion of the data presented, not because of its resemblance to any biological fluid but because certain of its properties make it ideal for the present studies. It is completely immiscible with Antarox A-400, and is well adapted for determination of the partition coefficient in an apparatus similar to that described by Hutchinson (2). The suitability of light liquid petrolatum was demonstrated by determining the partition coefficient for iodine between it and various solutions of iodine-potassium iodide. The partition coefficient between water and light liquid petrolatum was found to be 28–30 (25°) over the entire range of concentrations studied; it was then possible to calculate the amount of iodine (I_2) in the aqueous potassium iodide layer. In this way, the equilibrium constant for the reaction $I_2 + I^- = I_3^-$ was measured and found to check with the reported value of 1.47×10^{-3} (24).

It will be noticed (Fig. 5) that the addition of the third component (glycerin or Antarox) modified the distribution characteristics of iodine. The results are markedly different, however, since glycerin produces a gradual change which becomes pronounced in higher concentrations (above 10%); Antarox produces its nearly maximum effect in quite dilute solutions (about 1%). An increase in the concentration of Antarox will again show a decrease in partition coefficient in the extremely concentrated solutions. As a matter of fact, no true partition coefficient could be determined between 100% Antarox and light liquid petrolatum. Therefore, it may be concluded that in making comparisons, solutions with different concentrations of the surface-active agent (even when small differences and low concentrations are involved) have to be treated as different solvents. Any data comparing availability of a drug from two solvents are meaningless unless the functional relationship between the two solvents is also made known. The addition of a surface-active agent changes not only the rate of approach of an equilibrium but also the equilibrium itself.

Furthermore, the partition coefficient for iodine between chloroform and 10% glycerin in water is constant at about 130, regardless of the concentration of iodine. The analogous partition coefficient between light liquid petrolatum and 6% Antarox in water is dependent on the concentration of iodine. It is evident (Fig. 5) that the partition coefficient for a drug between a fixed concentration of surface-active agent and some other solvent will be a function of the concentration of the drug. This

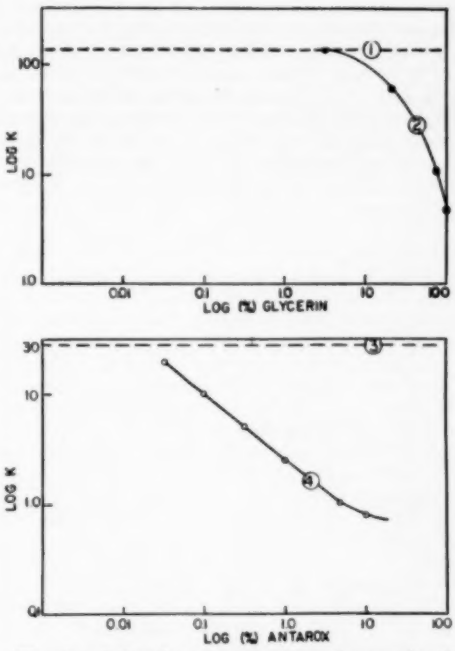

Fig. 5.—A log–log plot showing changes in the partition coefficient (K) of iodine with increasing concentrations of the added solvent.

1: $K = \dfrac{\text{Concn. in chloroform layer}}{\text{Concn. in water layer}}$

2: $K = \dfrac{\text{Concn. in chloroform layer}}{\text{Concn. in aq.-glycerin layer}}$

3: $K = \dfrac{\text{Concn. in light liquid petrolatum layer}}{\text{Concn. in water layer}}$

4: $K = \dfrac{\text{Concn. in light liquid petrolatum layer}}{\text{Concn. in aq -Antarox layer}}$

fact limits the usefulness of the partition coefficient as an index of availability of drugs from solutions of surface-active agents.

Effect of Increase in Drug Concentration.—The oil-water partition coefficient is often a useful property of a system, and its correlation with permeability has frequently been emphasized (25–27). However, in testing antiseptics, the actual bacteriological testing still remains the best and only reliable method. In Fig. 6, the data reported by Bean and Berry (28) have been replotted to show the relationship between the concentration of a drug (benzylchlorophenol) and its biological response (mean death time for *E. coli*) for any fixed concentration of the surface-active agent (potassium laurate). Again it is found that once the capacity factor has been made constant (fixed concentration of the surface-active agent) the mean death time becomes a function of the concentration of benzylchlorophenol. The discontinuity is perhaps due to the fact that the total concentration of the benzylchlorophenol is plotted, instead of the concentration in water; for a fixed concentration of the surface-active agent, these two quantities may be related in a manner similar to that shown for the iodine-Antarox system (Fig. 4).

Since the solubilization data are often expressed as moles of drug solubilized per mole of surface-active agent (7), it is useful to follow the changes in the antibacterial activity of the drug with increasing concentration of the surface-active agent by maintaining a constant molar ratio between the drug and the surface-active agent. This approach has been followed by several workers but in each case interpretation is difficult, due to the inclusion of too many variables in their experimental procedure. When the molar ratio between the drug and the surface-active agent is kept constant, a change in either may or may not be accompanied by analogous changes in the per cent saturation and biological activity. The bacteriological data reported by Bean and Berry (28) illustrate this point well. Figure 7 is a plot of data calculated from their findings (the reader is referred to the original article for their method of plotting). In the portion of the experiment reported here, the molar ratio between the benzylchlorophenol and potassium laurate had been kept constant at 0.0666.

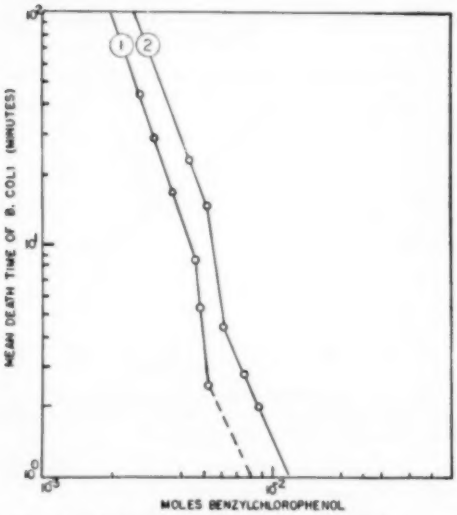

Fig. 6.—A log–log plot showing that the mean death time is a function of the concentration of the benzylchlorophenol (5-chloro-2-hydroxy diphenylmethane) only when the solvent is fixed. 1: Potassium laurate, 3.89×10^{-2} *M*; 2: Potassium laurate, 6.48×10^{-2} *M*.

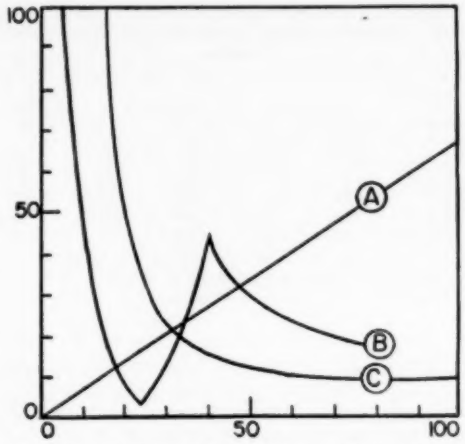

Fig. 7.—Ranges of killing time. Curve A: the change in moles of benzylchlorophenol (5-chloro-2-hydroxy diphenylmethane) $\times 10^4$ with potassium laurate $\times 10^4$ at a fixed ratio of 0.0666 *M*/*M*; Curve B: the change in mean death time for *E. coli* (minutes) with potassium laurate $\times 10^4$, maintaining the same molar ratio as in curve A; Curve C: the change in per cent saturation of the total solution with increasing potassium laurate $\times 10^4$, maintaining the same molar ratio as in curve A.

It will be noticed that the plot of killing time can be divided into the following three ranges:

1. A range of rapid decrease in killing time or increase in the amount of drug reacting with *E. coli* per unit time.
2. A range of increase in killing time.
3. A range of gradual decrease in killing time.

The first range starts from the zero concentration of potassium laurate and benzylchlorophenol. The amount of benzylchlorophenol required to saturate water at 20° is 157 mg./L.; under the conditions of the experiment, this quantity gave a killing time of more than 500 minutes.[7] In the presence of a small amount of potassium laurate below the concentration necessary to form micelles (approx. 0.025 *M*) and even below the concentration neces-

[7] It should be mentioned here that Bean and Berry have reported the water solubility to be 1 part in 15,000. However, in this laboratory, the water solubility was determined by using a Cary spectrophotometer. The molecular extinction was found to be 2,360 in water at 282 mμ and 3,765 in dilute alkali or dilute potassium laurate (10^{-3} *M*) at 298 mμ. The solubility in water was 1 in 6,300; in dilute alkali or potassium laurate (less than 10^{-3} *M*) it was 1 in 2,480. This increase in solubility in dilute alkali or potassium laurate is expected from the weak acidity of benzylchlorophenol (its dissociation constant should be approximately the same as that of *p*-chlorophenol which has a dissociation constant of 2×10^{-9} (29).

sary to cause solubilization (Fig. 8), the killing time is reduced from more than 500 minutes to about two minutes. This fact alone strikingly illustrates that under certain conditions a surface-active agent may convert a seemingly inactive drug (showing no activity by the method of testing) into a strikingly active one. The reduction in killing time continues with increasing concentration of potassium laurate (and consequently benzylchlorophenol) until the micelles begin to form. In the first range, a small amount of surface-active agent reduces the surface and interfacial tension between the bacteria and the surrounding medium, favoring an increase in adsorption of benzylchlorophenol and leading to the observed enhancement of the activity.

The second range of killing time starts approximately from the concentration at which micelles form (CMC). At this concentration, the avid lipoid-solubilizing effects of the micelle begin to deplete the aqueous environs of benzylchlorophenol. This additional factor causes a shift in the adsorptive capacities of the drug. Thereafter, even though the total amount of benzylchlorophenol is being increased gradually (curve *A* in Fig. 7), per cent saturation decreases rapidly. As noted earlier, the reduction in per cent saturation leads to a reduction in the thermodynamic activity of benzylchlorophenol which is responsible for the observed increase in killing time.

The third range or region of declining killing time starts from a sudden reversal in curve *C* of Fig. 7. Bean and Berry explain this reversal by postulating a multilayer adsorption of the micelles on the bacterial surface. No proof was presented to support this assumption, however, and evidence may be cited to show that multilayer formation is an extremely unlikely event for anionic colloidal electrolytes (22, 30). Undoubtedly, benzylchlorophenol is suddenly able to adsorb more avidly upon bacteria, even though the drug concentration in the aqueous phase must concomitantly decrease with the continually decreasing percentage saturation of the solution. Whether a new, highly adsorptive complex is formed with potassium laurate or with its hydrolysis products is not known, but the approximate coincidence of this biological event with the point of discontinuity in the solubilizing curve (Fig. 8, 0.03 *M* potassium laurate) is suggestive of a more complicated situation. The exact form of the micelle as well as the possibility of different types of micelles is not clearly understood at this time. Without further knowledge of the physical distribution and adsorptive characteristics, the abrupt change in activity cannot be explained, but it clearly illustrates the complicated (and possibly unknown) factors in certain surface-active systems.

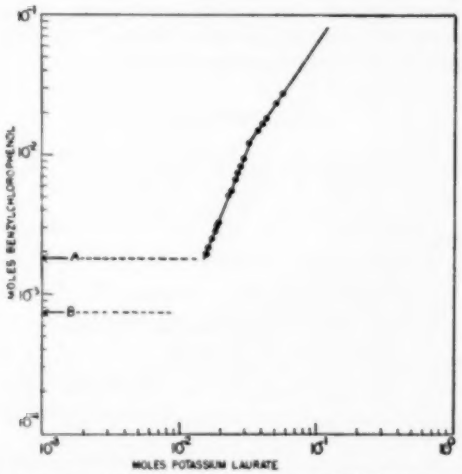

Fig. 8.—A log–log plot of the solubilization of benzylchlorophenol (5-chloro-2-hydroxy diphenylmethane) in moles/L. by solution in potassium laurate (moles/L.) at 20°. A: saturation solubility of benzylchlorophenol at *p*H 9–10; B: saturation solubility of benzylchlorophenol in distilled water.

Unique Effects (17, 22, 31, 33–35).—This discussion has been directed toward the influence of surface-active agents upon the driving force determining the various forms of availability defined earlier. There are certain other influences of surface-active agents which may or may not be consequential, depending upon the type of availability measured.

It was mentioned that in low concentrations of surface-active agents, an enhancement of the drug action is observed. This is due to the surface properties of the surface-active agent which are quite distinct from the bulk properties involved in micelle formation. The same point is well illustrated by the work of Alexander and Trim (6). They found that addition of small amounts of cetyl trimethyl ammonium bromide (CTAB) caused enhancement in the rate of uptake of hexylresorcinol by the Ascaris worm. This enhancement continued until the critical micelle concentration was reached, at which point there was a rapid decrease in the rate of uptake of hexylresorcinol by the Ascaris worm. These findings were followed, together with the changes in the interfacial tension between mineral oil and water with the addition of CTAB. The analysis of the surface tension data combined with the effects of CTAB on adsorption of hexylresorcinol indicates that the presence of small amounts of the surface-active agent favors an increase in adsorption of hexylresorcinol at the interface leading to an increased absorption by the Ascaris worm. Furthermore, as Alexander and Trim pointed out, there is evidence to show that small amounts of CTAB changes the permeability of Ascaris and may be responsible for the marked increase in the initial uptake. This change is especially typical of cationic surface-active agents and will be observed only if the permeability is being followed. It is only under those conditions that a correction for it need be made.

It should be mentioned here that this type of enhancement of the action of antimicrobial agents is not restricted to cationic agents. Antarox A-400, a completely innocuous agent which maintains microbial growth in all concentrations, can be shown to cause an enhancement of the activity of iodine, even though this drug acts on the surface of the bacteria (36). Phenol in Aerosol MA seems to have a similar action, as shown by Alexander (22). These enhancements, especially noticeable in low concentrations, may be due to the detergent action of the surface-active agent on the

bacterial cell, reduction in the interfacial tension leading to greater rate of adsorption, or complexing of some agent in bacterial environment (37).

Certain surface-active agents, especially cationic, have their own antibacterial activity and application in bacteriology (32). When these agents are used in formulation with other antiseptics (34), it becomes a difficult problem to separate the action of two antibacterial agents. In the following papers, examples will be presented to show the possibility of predicting whether or not the two will be synergistic, antagonistic, or merely additive.

Certain drugs solubilized by surface-active agents may gain (or lose) activity in contact with biological systems. These changes are brought about by the proteins and other constituents with which they may come in contact. Anionic and cationic agents possess charges which allow them to interact with the proteins in the environment being tested (38). This interaction will undoubtedly upset the micellular equilibrium of the system and cause a change in the solubility or availability of the drug being applied. Such reaction may be an important factor in skin surface sterilization such as has been shown for hexachlorophene (G-11) (39–40). Figure 2 shows the solubilization of this drug in a nonionic surface-active agent. Although quantitative data may not be presented, analogous solubilization is found for this drug in anionic surface-active agents such as are used in the commercial formulations available for presurgical detergency. Upon contact with the hands and skin proteins, it is probable that both the drug and micellular solution and the anionic surface-active agent may adsorb to a great extent. The adsorption of the anionic surface-active agent and subsequent rupture of the micelle allows for the release of the G-11.

Pharmaceutical Application.—From this discussion, therefore, it may be concluded that when water is used as a solvent for a slightly soluble drug, even if the initial per cent saturation is high, the amount of drug reacting with bacteria and the surrounding medium reduces the activity rapidly. On the other hand, when drug is used in a solution of a surface-active agent with the same initial per cent saturation, the amount used by the bacteria changes the per cent saturation only slightly, due to the high capacity of the solution. The micelles in the solutions of surface-active agent, therefore, serve as a storehouse, and an antiseptic agent whose water solution is normally inactive may seem to develop new activity.

It is necessary that a concentration of the surface-active agent be used in which the drug is neither so soluble that an extensive amount is required to produce saturation nor so insoluble that a small loss will lower the per cent saturation until the solution becomes ineffective.

Since solutions of surface-active agents are not simple solvents and solubilization is not analogous to simple solution, every drug and surface-active agent will show an optimum which can be determined only if both biological and physical data are available. Furthermore, due to the nature of the solutions of the surface-active agent, the absolute amount of drug present in the solution is not the measure of the amount of the drug biologically available. Hampil's data (20) seem to illustrate this point; 0.6% phenol is completely ineffective in 5% sodium oleate but quite effective in 2% sodium oleate for killing bacteria, and 0.5% phenol is quite effective in 1% sodium myristate but almost ineffective in 1% sodium oleate. This observed fact could have been predicted from the solubilization data, since it is known that much larger amounts of phenol are required to saturate 1% sodium oleate than to saturate 1% sodium myristate.

The surface-active agents possess intrinsically hydrophobic parts in their molecules, and when mixed with water or another vehicle, they change the character of the vehicle and the body fluids. The change in the partition coefficient may be so pronounced and adverse that the usefulness of the vehicle may become questionable. The use of surface-active agents to solubilize drugs in ointment bases and suppositories offers an example. Most suppository and ointment bases are lipoidal in character and the availability of any drug from these preparations must be carefully investigated (41).

SUMMARY

1. A preliminary report is given which shows a definite need for appreciation of factors involved in evaluating biological data on antimicrobial agents in solutions of surface-active agents. The lack of such data, together with biological findings in recent literature, makes the comparison impossible, and in some cases the results are actually irreproducible.

2. In this paper it has been pointed out that changes in the concentration of the antibacterial agent are not an index of the changes in the antibacterial activity of solutions containing surface-active agents.

3. It has also been noted that changes in the concentrations of the surface-active agent modify the thermodynamic activity which is the real measure of the availability of the drug.

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EIGHTH INTERNATIONAL BOTANICAL CONGRESS

It is announced that the Eighth International Botanical Congress will be held in Paris from July 2–14, 1954. The Congress will consist of plenary sessions and of seminars devoted to special subjects of less general interest.

Excursions are planned to take place before, during, and after the Congress. From June 15–20, excursions are scheduled to North Africa, the Languedoc, Alsace, Lorraine, Brittany, Normandy, the Vendee and the Basque provinces. From July 2–14, excursions will be restricted to the environments of Paris. From July 15–22, trips will be made from Paris to Nice via Burgundy, the Jura, the Alps, the Provence, and the Pyrenees. A follow-up Congress will be held in Nice from July 22–26, to be succeeded by trips to Corsica, Tropical Africa (the Senegal and Mauritania), and to Equatorial Africa (the Ivory Coast and Guinea).

The attendance fee will probably be Frs. 5,000 ($15), with reduced rates for students and persons accompanying members of the Congress. Hotel reservations will be guaranteed from the most luxurious (in large hotels) to the most modest (in school dormitories). The detailed program of the Congress is in press and will be sent to all botanists, agronomists, foresters, etc., whose addresses are known. Inquiries should be sent to the Secretary General of the VIIIth International Congress of Botany, 292 rue Saint-Martin, Paris, 3e, France.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Molybdenum carbonyl
Isophthalaldehyde
Thiobenzoic acid
3,4,5-Trichlorobenzaldehyde
Methyl α-benzylacrylate
Trinaphthylmethane
3-Fluorophenylalanine
3,3-Dichloropropene-1
2-Cyanobutadiene-1,3
2,6-Dimethylheptanetriol-2,4,6
5,5′-Dichloro-6,6′-dimethylindigo
1-Methyl-4-methylolglyoxaline
1-Methyl-5-cyanomethylimidazole
2,6-Dichlorodiphenylamine
Camphane aldehyde
Vulbocapnine
Hypertensinogen
Perillaldehyde
Quercetagetin
Daidzin

The Six-Point Design in the U. S. P. Microbiological Assay of Vitamin B_{12}*

By J. A. CAMPBELL, J. M. McLAUGHLAN, JEAN A. CLARK, and C. W. DUNNETT

Since few published data were available concerning the precision of the U. S. P. method for Vitamin B_{12}, a detailed statistical study was undertaken to assess the importance of factors affecting the response. On the basis of a linear relation between the logarithm of dose and the logarithm of response, the six-point design (three doses of standard and three doses of unknown) was applied to this assay. The design was found to have several advantages, and under well-controlled conditions it may be simplified still further. The method was precise and reproducible. The linear relationship between log dose and log response was valid, and was not influenced by the type of product being assayed, e. g., liver extract injectable or vitamin B_{12} concentrate. The response to vitamin B_{12} was influenced by the preparation of tubes and by their position during autoclaving and incubation. To avoid possible bias caused by these factors, the tubes were randomized before addition of the media.

SINCE the discovery that Vitamin B_{12} is an essential growth factor for certain microorganisms (1, 2), many phases of the microbiological assay of this vitamin have been investigated. Methods employing a number of different microorganisms have been described (2–9) and there appear to be differences of opinion as to which method is most suitable (10). The second collaborative assay conducted by the United States Pharmacopeia showed that the U. S. P. procedure (11) employing *L. leichmannii* A. T. C. C. 7830 in a seventy-two hour titrimetric assay yielded results which were reproducible from one laboratory to another but which varied significantly in precision (12, 13).

Several authors (14–16) have pointed out the desirability of transforming the response to plot as a straight line against the logarithm of the dose. Instability of response, i. e., changes in the shape of dose-response relationship, will be evident on such a plot. Gross dissimilarities in response of standard and unknown can readily be detected by a comparison of the slopes of the two response lines. This is of particular importance in the microbiological assay of substances like vitamin B_{12} which often occurs (in liver extracts and concentrates) in the form of a mixture of two or more vitamin B_{12} factors, while the standard is crystalline vitamin B_{12}. For a linear relationship with the logarithm of the dose, the six-point design (three doses of standard and unknown) with the doses spaced geometrically, i. e., at equal log dose intervals, makes efficient use of a given number of tubes (14,15) and at the same time permits tests of linearity and parallelism. Furthermore, the relative potency of the unknown and standard preparations can be easily determined graphically from the horizontal distance between two parallel lines fitted to the original data plotted directly on log-log paper.

Preliminary studies in this laboratory of the U. S. P. procedure for assaying vitamin B_{12} indicated that the logarithm of the response usually plotted as a straight line against the logarithm of the dose (17). A typical response for the U. S. P. procedure is shown in Fig. 1 on three different plots: arithmetic, arithmetic-log, and log-log. The upper part of the response curve on a log-arithmetic basis (*B*) is approximately a straight line, as Brownlee has noted (12). It is evident on the log-log plot (*C*) that the entire range from 0.02 to 0.10 mγ is linear.

At about the same time, a paper by Flynn, *et al.* (18), also indicated a linear response on a log-log basis in a turbidmetric assay for vitamin B_{12}; more recently Emery, *et al.* (19), reported the use of a short linear range on a log dose-arithmetic response plot. Brownlee (12) reanalyzed the data of the U. S. P. collaborative assay, using only four points on this latter basis, and found that this type of procedure was more efficient than the traditional assay. Later, Brownlee and Lapedes (20) studied sources of error in the turbidmetric assay, utilizing a linear response on a log-arithmetic basis and a four-point design.

The object of this paper is to present the results of studies on the U. S. P. XIV method which has been modified by employing the six-point design on a log dose-log response basis. Under proper conditions, it is a relatively precise method and the suggested design permits considerable simplification.

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PROCEDURE

An experiment was set up using the six-point design to study the reproducibility of the method and the effect of possible sources of variation. Four samples, which included two liver extracts and two vitamin B_{12} concentrates, were assayed on each of five days. Samples A and B were liver extracts injectable, 15 units per cc., and samples C and D were vitamin B_{12} concentrates (fermentation source). In an attempt to minimize deterioration of samples during the course of this experiment, first dilutions were prepared in 25% alcohol, stored in the cold, and used for each assay.

The assay procedure was essentially the same as the U. S. P. method (11), but the design and technique differed in a number of respects. Three dosage levels were used for both standard and unknown preparations. The levels 0.02, 0.04, and 0.08 mγ of vitamin B_{12} were selected as representing the best spacing of dosage. These concentrations corresponded to the 1.0, 2.0, and 4.0-ml. levels in the U. S. P. procedure. Finney (21) has shown that the greatest efficiency in multiple unknown assays is obtained when the number of replications on the standard and unknown preparations are in the ratio of the square root of the number of unknown preparations. Thus, since four unknowns were tested, eight tubes were set up at each of the three levels of the standard and four tubes were used at each level of the unknown. Tubes of 16-mm. diameter were used instead of the recommended 20-mm. size. The inoculum was centrifuged and resuspended twice before diluting (1:5) for use.

All tubes were randomized within the rack just before addition of the basal medium. They were kept in this order during autoclaving, incubuation, and titration, so that any effect caused by addition of the medium, autoclaving, incubation, or titration would be distributed in a random manner over all levels and samples. (The effect of randomization is discussed in more detail later.) Titrations were made with bromthymol blue as indicator. No difficulty was experienced with the end point in any of the samples tested, and Brownlee (12) has indicated that this type of titration is more precise than the electrometric technique. As Loy, *et al.* (22), have pointed out, the glassware, particularly the tubes, must be scrupulously clean. It has been found that treatment of the tubes for two hours with 1 *N* NaOH, followed by rinsing 10 times with water, will remove any interfering material.

RESULTS

Since the logarithm of the response was found to plot as a straight line against the logarithm of the dose, the responses in their original units were changed to logarithms. The data in logarithmic units of a typical assay (#2) are shown in Table I.

An analysis of variance was made on each assay and the results have been compiled in Table II. It may be noted that there was a significant curvature in only one assay (#4). In the remaining four assays, the data plotted satisfactorily as straight lines. Only in one assay (#1) was there a significant variation in slope among the five vitamin preparations.

Variation at Each Dosage Level.—In terms of logarithms there was a tendency toward less variation among the replicates at the high dosage levels than at the lower levels. Bartlett's test for homogeneity of variances (23) showed that the variation between the mean squares for replicates at the three levels was significant for assays #3 and #4. In his analysis of the recent collaborative study on the U. S. P. method for vitamin B_{12} assay, Brownlee (12) found that the variance was substantially independent of the dosage level when the response was expressed in its original units. However, for the data reported here, there appeared to be a tendency for more variation between the replicates at the higher levels when the response was measured in original units. In fact, in original units of response, assays #2 and #5 showed significant differences in mean squares at the three levels, as judged by Bartlett's test.

Finney (24) has described a general procedure for handling assays in which the variance is not independent of the dosage levels, which assigns appropriate weights to the responses, and Bliss (25) has described in detail the particular procedure that would be applicable if the variance were assumed to be independent of the dosage level when the response is measured in original units. Rather than apply the weighted method of Finney or Bliss to assays #3 and #4 and perhaps to assay #1, and the usual unweighted method to assay #2 and #5, the

TABLE I.—LOG RESPONSE DATA FOR A TYPICAL ASSAY (#2)

Preparation	Dose, Ml.	Log Response								Sum	Range
Std.	1	0.628	0.580	0.556	0.628	0.633	0.591	0.602	0.562	4.780	0.077
	2	0.763	0.767	0.720	0.748	0.752	0.732	0.748	0.785	6.015	0.065
	4	0.922	0.892	0.881	0.922	0.898	0.895	0.929	0.903	7.242	0.048
A	1	0.591	0.556	0.550	0.580	...	...	...	...	2.277	0.041
	2	0.690	0.708	0.720	0.732	...	...	...	...	2.850	0.042
	4	0.854	0.869	0.906	0.886	...	...	...	...	3.515	0.052
B	1	0.597	0.633	0.591	0.585	...	...	...	...	2.406	0.048
	2	0.724	0.756	0.792	0.799	...	...	...	...	3.071	0.075
	4	0.881	0.922	0.924	0.908	...	...	...	...	3.635	0.043
C	1	0.613	0.643	0.633	0.633	...	...	...	...	2.522	0.030
	2	0.778	0.820	0.826	0.816	...	...	...	...	3.240	0.048
	4	0.927	0.927	0.968	0.968	...	...	...	...	3.790	0.041
D	1	0.628	0.653	0.658	0.643	...	...	...	...	2.582	0.030
	2	0.813	0.782	0.782	0.810	...	...	...	...	3.187	0.031
	4	0.944	0.932	0.940	0.968	...	...	...	...	3.784	0.036

TABLE II.—ANALYSIS OF VARIANCE FOR INDIVIDUAL ASSAYS

Source of Variation	D. F.	Mean Squares for Assay No. 1	2	3	4	5
Preparations	4	0.005126[a]	0.012877[a]	0.008755[a]	0.013049[a]	0.008137[a]
Levels	2	0.208948[a]	0.570392[a]	0.354402[a]	0.564526[a]	0.393677[a]
Slope	1	0.417760[a]	1.140525[a]	0.708102[a]	1.126081[a]	0.787200[a]
Curvature	1	0.000136	0.000259	0.000702	0.002970[b]	0.000154
Preparations × Levels	8	0.000235	0.000247	0.000184	0.000159	0.000398
Parallelism	4	0.000384[b]	0.000070	0.000304	0.000164	0.000060
Prepar. × curv.	4	0.000086	0.000425	0.000065	0.000154	0.000736
Replicate Tubes	57	0.000139	0.000472	0.000177	0.000353	0.000708
Low level	19	0.000179	0.000526	0.000292	0.000567	0.000726
Middle level	19	0.000164	0.000514	0.000171	0.000374	0.000724
High level	19	0.000075	0.000376	0.000069	0.000118	0.000673

[a] Significant at the level $P = 0.01$.
[b] Significant at the level $P = 0.05$.

TABLE III.—COMPARISON OF SOME STATISTICS FOR THE INDIVIDUAL ASSAYS[a]

Statistic	Assay No. 1	2	3	4	5	Chi-square
Potency of A	0.84 (8.4)	0.87 (8.7)	0.82 (8.2)	0.82 (8.2)	0.84 (8.4)	2.00
Potency of B	1.02 (15.3)	1.04 (15.6)	1.02 (15.3)	1.02 (15.3)	1.04 (15.6)	0.34
Potency of C	1.22 (36.6)	1.22 (36.6)	1.22 (36.6)	1.21 (36.3)	1.21 (36.3)	0.14
Potency of D	1.21 (24.2)	1.22 (24.4)	1.15 (23.0)	1.14 (22.8)	1.14 (22.8)	3.52
Combined slope, b	0.310	0.512	0.403	0.509	0.425	...
Standard deviation, s	0.0118	0.0217	0.0133	0.0188	0.0266	...
Index of precision, s/b	0.0381	0.0424	0.0330	0.0369	0.0625	...
Range estimate of s, s_R	0.0116	0.0213	0.0126	0.0175	0.0269	...

[a] Figures in parentheses indicate potency in μg. per ml. Samples A and B were liver extracts injectable and samples C and D were B_{12} concentrates from fermentation sources.

unweighted method was used for all five assays to preserve uniformity and simplicity. Whichever method of calculation is used, substantially the same values will be obtained for the potencies. For example, in assay #4, which appears to be the most favorable one of the five for applying the weighted method, the four potency values by this method are 0.83, 1.02, 1.25, and 1.16, which may be compared with the values found by the unweighted method (Table III).

Variation from Day to Day.—In Table III various statistics, which have been calculated from the data of each of the five assays, are given for comparative purposes. Substantially the same potencies were obtained for each of the four vitamin preparations in all five assays, and none of the values of Chi-square for testing the day-to-day variation (25) were significant. The slope, b, varied considerably from day to day, and this variation is shown to be significant (in Table IV). The standard deviation, s, calculated as the square root of the mean square for replicates from the analysis of variance tables in Table II, is also shown. It varied considerably from day to day, and the variation was judged significant by Bartlett's test (23). The ratio $s/b = \lambda$ varied from 0.033 to 0.063. The standard error of the logarithm of the potency, s_M, is given by the approximate formula [for the exact formula, see Bliss (25), p. 505]:

$$s_M = \frac{s}{b}\sqrt{\frac{1}{N_s} + \frac{1}{N_u}}$$

which, for $N_s = 24$ and $N_u = 12$, varied from 0.012 to 0.027 for the five assays. These are equivalent to ratios of 1.05 and 1.11, respectively, or potency limits of error of ±5 and ±11% at $P = 0.05$, indicating that the assay method reported here is considerably more precise than the turbidimetric method for which error limits given by the ratio 1.18 are reported by Brownlee and Lapedes (20), and the U. S. P. method which had an average ratio of 1.28 as reported by 23 collaborating laboratories (12).

Estimates of the standard deviation, based on ranges for each assay, are given in the last line of Table III. In all cases the range estimates agreed closely with the analysis of variance estimates, and it is suggested that the validity tests and the calculation of the error limits for each assay could be carried out much more quickly using the range estimate of standard deviation instead of carrying out the complete analysis of variance. The required calculations, shown in Table V, are discussed below.

Combined Analysis.—In Table IV, the combined analysis of variance of all five assays is shown Since the mean squares for replicates in the five individual assays were not homogeneous (as judged by Bartlett's test), the F tests in this table, which utilize a pooled estimate of the variation between replicates as error, are not exact. However, the approximation can be improved by means of Satterthwaite's formula (26) which alters the number of degrees of freedom for error from its nominal value of 285 to its "effective" value of approximately 217 in this example. The change has only a slight effect on the critical values to be used for the F ratios.

TABLE IV.—COMBINED ANALYSIS OF VARIANCE

Source of Variance	D. F.	Mean Squares	F
Assays	4	0.166015	...
Preparations	4	0.046258	...
Assays × preparations	16	0.000422	1.14
Levels	2		
Slope	1	4.057560	...
Curvature	1	0.001066	2.88
Assays × levels	8		
Assays × slope	4	0.030527	82.5[a]
Assays × curvature	4	0.000789	2.13
Preparations × levels	8		
Prep. × slope	4	0.000021	0.06
Prep. × curvature	4	0.000836	2.26
Assays × prep. × levels	32		
Assays × prep. × slope	16	0.000240	0.65
Assays × prep. × curv.	16	0.000157	0.43
Replicate tubes	285[b]	0.000370	...
Total	359		

[a] Significant at the level $P = 0.01$.
[b] As explained in the text, the "effective" number of degrees of freedom should be taken as 217 rather than 285.

It may be noted that the mean squares for "assays × slope" was highly significant, reflecting the fact that the slope of the response lines was not constant from day to day. The mean squares for "preparations × slope" and "assays × preparations × slope" were not significant, indicating that on any one day the slopes of the response lines for the various preparations were parallel, as required for a valid method of assay. This demonstrated that all samples, regardless of the type of vitamin B_{12} which they contain, were reacting in a manner similar to that of crystalline vitamin B_{12}. None of the effects for curvature, "assays × curvature" or "preparations × curvature," was significant.

Range Method of Calculation.—In Table V are shown the complete calculations, based on the range estimate of standard deviation, for the estimation of potencies and their limits of error. The formula used for the range estimate of the standard deviation is that suggested by David (27). A slightly different formula has been proposed by Grubbs and Weaver (28). A table of the factors required to calculate the range estimate of standard deviation is given in Table VI which is a condensation of more extended tables given elsewhere (27, 28). The formula used for s_M has been simplified by dropping a term, whose value was negligible, from the formula usually used, e. g., Bliss (25). If it is desired to test for parallelism and curvature, the regular tests may be carried out using the range estimate of standard deviation.

The formulas in Table V represent a simplification of the approach usually taken by an analysis of variance. As shown in Table III, estimates of error determined from the range agreed very well with those obtained by an analysis of variance. Complete data on the assay may be summarized as shown at the foot of Table V. Such a scheme permits estimation of the precision of an assay with a minimum of time and effort.

Effect of Position of Tubes.—Brownlee and Lapedes (20) found an effect of position of tubes in the turbidimetric assay for B_{12} and it has been noted in this laboratory that tubes near the outside of the rack tend to titrate to a higher value than the tubes in the center. It was therefore decided to set up an experiment to determine whether this variation was caused by autoclaving, incubation, or preparation of the tubes.

One hundred tubes were numbered consecutively and handled (i. e., for adding medium, inoculum, etc., and for titrating) in that order in sets of 20.

TABLE V.—SHORT METHOD OF CALCULATION OF POTENCY AND CONFIDENCE LIMITS BASED ON RANGE AND ILLUSTRATED ON THE DATA IN TABLE I

Total range for standard, $R_s = 0.190$; total range for unknowns, $R_u = 0.517$

$$s_R = \frac{p_s R_s + p_u R_u}{3q_s + 3Kq_u + 1/2} = \frac{4.24(0.190) + 2.66(0.517)}{3(12.06) + 12(5.48) + 0.50} = 0.0213$$

where
p_s, p_u, q_s, and q_u are taken from Table VI.
K = No. of unknown preparations = 4.
r = No. of replicates = 4, 8, or 24 depending on whether one of the unknowns, the standard, or all preparations are being considered.
L = Sum of all responses at high level − sum of all responses at low level = 21.966 − 14.567 = 7.399.
i = Log dose interval = 0.301.

Combined slope, $b = \frac{L}{2ri} = \frac{7.399}{48(0.301)} = 0.5121$.

$$s_M = \frac{s_R}{b}\sqrt{\frac{1}{N_s} + \frac{1}{N_u}} = \frac{0.0213}{0.5121}\sqrt{\frac{1}{24} + \frac{1}{12}} = 0.0147$$

$t = 2.007$ [$P = 0.05$; d. f. = $1/2(3q_s + 3Kq_u) = 51$]; $ts_M = 0.0295$.

Prep.	Mean Response, $\bar{Y}$	$M = \frac{\bar{Y}_u - \bar{Y}_S}{b}$	Potency Antilog M	$M \pm ts_M$	Potency Limits, Antilog $(M \pm ts_M)$
Std.	0.752	....	...	.....	.....
A	0.720	−0.0625	0.87	−0.0920, −0.0330	0.81–0.93
B	0.759	0.0137	1.03	−0.0158, 0.0432	0.96–1.10
C	0.796	0.0859	1.22	0.0564, 0.1154	1.14–1.30
D	0.796	0.0859	1.22	0.0564, 0.1154	1.14–1.30

The tubes were placed in two racks for autoclaving according to the predetermined scheme shown in Table VII. After autoclaving, the tubes were reassigned to new positions, also shown in Table VII, for incubating. After the incubation period, they were titrated in the order in which they were numbered.

It may be noted that each block of 25 tubes in the rack consisted of 5 tubes from each of the 5 sets in which the tubes were divided for handling. For example, the block of tubes in the upper half of the left-hand rack contains tubes 1–5, 21–25, 41–45, 61–65, and 81–85. Moreover, the five tubes from any one set were distributed in the block so that one of them was assigned to each row and also to each column of the block. For incubating, the tubes within each block were rearranged so that each row and each column of the block again received one tube from each set, and also one tube from each row and from each column of the block arrangement used for autoclaving. Except for these restrictions, the assignment of the tubes was a random one. To achieve this, the arrangement for each block conformed to a hyper-greco-latin square design (29). Thus, the effects of sets in preparation of tubes, of rows and columns in autoclaving, and of rows and columns in incubating were all balanced within each of the four blocks. After incubation the growth of the organism was measured by titrating the tubes in the same order in which they were prepared.

Analysis of variance was made on the data of each block of 25 tubes, and the four sets of data were then combined with the results shown in Table VIII. It is evident that the autoclave and the incubator both have an effect on the titration values obtained; however, the effect of the incubator appears to be rather more important. The significance of the factor "sets of tubes" indicates that there is a difference between tubes caused by variation either in the medium or in titration, or both. It is considered that most of the effect is probably caused by slight changes in the medium. The fact that the interaction "sets × blocks" is not significant indicates that the trend is uniform within each set of tubes. The significance of these sources of variation is such that they cannot be disregarded for they will have a very definite effect on the assay. Randomization of tubes after preparation distributes the effects of such factors over all dosage levels of all samples and removes the possibility of bias affecting a single sample adversely.

DISCUSSION

The six-point design has been in use in this laboratory for more than a year. In practice it has been found to have several advantages over the conventional type of assay recommended by the United States Pharmacopeia. The six-point design is simpler to set up and fewer tubes are required. For those interested in critical studies of the precision of the method, it permits tests of precision, validity, linearity, and parallelism. A convenient method of calculating confidence limits has been illustrated.

The method itself may be simplified still further if desired. For graphical estimation of potency it is as convenient to use log-log paper as the arithmetic scale and it is easier to draw and compare straight

TABLE VI.—FACTORS FOR ESTIMATING STANDARD DEVIATION FROM RANGE

No. of Replicates, r	p	q
2	1.55	1.75
3	2.15	3.63
4	2.66	5.48
5	3.12	7.25
6	3.52	8.93
7	3.90	10.54
8	4.24	12.06
9	4.55	13.51
10	4.84	14.90

TABLE VII.—DESIGN OF EXPERIMENT TO STUDY FACTORS AFFECTING THE RESPONSE IN VITAMIN B_{12} ASSAY[a]

(A)

Rack Positions for Autoclaving

1	62	83	44	25	71	92	15	54	33
85	3	24	61	42	32	14	51	73	95
43	21	65	82	4	55	31	93	12	74
64	45	2	23	81	13	75	34	91	52
22	84	41	5	63	94	53	72	35	11
70	47	88	26	9	56	37	80	99	18
46	29	67	8	90	38	19	57	76	100
7	86	30	69	48	97	78	16	40	59
89	68	6	50	27	20	96	39	58	77
28	10	49	87	66	79	60	98	17	36

(B)

Rack Positions for Incubating

81	5	62	24	43	51	94	75	12	33
44	21	85	63	2	13	31	54	95	72
65	42	23	1	84	92	73	11	34	55
22	83	4	45	61	35	52	93	71	14
3	64	41	82	25	74	15	32	53	91
6	28	47	69	90	99	38	60	16	77
29	50	66	88	7	57	96	18	79	40
87	9	30	46	68	36	80	97	58	19
70	86	8	27	49	20	59	76	37	98
48	67	89	10	26	78	17	39	100	56

[a] Numbers indicate order of preparation of tubes.

TABLE VIII.—ANALYSIS OF VARIANCE OF FACTORS AFFECTING THE RESPONSE TO VITAMIN B_{12}

Source of Variation	D. F.	M. S.	F
Blocks	3	0.02910	1.48
Rows within blocks in autoclave	16	0.05219	2.66[a]
Columns within racks in autoclave	8	0.05069	2.58
Autoclave columns × blocks	8	0.04881	2.49
Rows within blocks in incubator	16	0.06438	3.28[a]
Columns within racks in incubator	8	0.12206	6.22[b]
Incubator columns × blocks	8	0.00406	0.20
Sets of tubes	4	0.19542	9.96[b]
Sets × blocks	12	0.02877	1.47
Remainder	16	0.019625	...
Total	99		

[a] Significant at $P = 0.05$.
[b] Significant at $P = 0.01$.

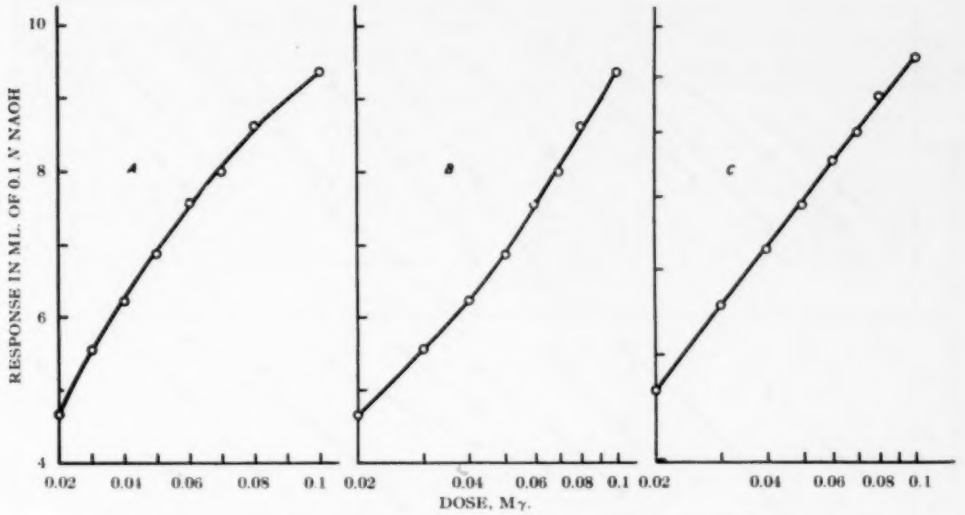

Fig. 1.—Response in U. S. P. microbiological assay for vitamin B_{12} plotted in 3 ways.
A: arithmetic dose–arithmetic response
B: log dose–arithmetic response
C: log dose–log response

lines than curves. If the response is found to be linear and stable from one assay to another, the four-point design, as suggested by Brownlee (12), may be used. Experience in this laboratory has indicated that in most assays the responses of standard and unknowns are parallel, and it may therefore be preferable to use three doses of standard and a single dose of each sample. Three doses of the standard are considered necessary to detect the occasional nonlinear assay. As the single unknown dose would correspond to the middle dose of the standard, a sample could vary from 50 to 200% of expected potency and still be within the response range of the standard. Results of assays carried out on such a basis have been found to agree closely with those obtained from assays involving three points for each sample.

The log-log transformation appears to have rather wide application. It has been learned recently that several laboratories have been using this transformation for plotting their data. It is interesting to note that the data of Loy, Haggerty, and Kline (30) plot as a straight line on log-log paper. The fact that other laboratories obtain such results would suggest that this is a true characteristic of the U. S. P. assay as presently specified. The work of Flynn, *et al.* (18), suggests that this relationship may be typical of many microbiological assays. Omission of tomato juice in the U. S. P. medium does not affect the linearity of response.

The data described above indicate that under proper conditions the U. S. P. method is relatively precise compared with some other procedures described in the literature. The turbidimetric assay (19, 20) has been reported to have an error of ±15 to ±18% ($P = 0.05$). In this laboratory, experience over a period of many months, using the six-point design in the U. S. P. method, has indicated that the limits of error vary from ±5 to ±10% ($P = 0.05$) and that more assays approach the 5% level of precision than the 10% level. There appears to be no question that the results of repeated assays are reproducible within the limits indicated by the internal error of individual assays. The procedure is valid for the estimation of the vitamin B_{12} activity of liver extracts and vitamin B_{12} concentrates obtained from fermentation sources.

Although lack of parallelism is occasionally found, data from assays of a large series of samples have shown no consistent trend in one direction for any particular sample. It has also been found that, while curvature appears in occasional assays, it is as often in one direction as the opposite and that, on the whole, the linear relation best fits the data. Figure 2 shows further data on a series of 22 standard response lines. In the upper half of the figure a series of assays is plotted in which the tubes were not randomized, while in those in the lower half the tubes were randomized. Only two response lines in this series of unselected assays were seriously nonlinear. Lack of randomization did not appear to contribute to nonlinearity. Those assays with slight curvature have been found to be reliable and Finney (31) has shown that the six-point design gives reasonably consistent results even when response curves are appreciably nonlinear. Wood (32) has indicated that the four-point design is also reliable in these circumstances.

It has been shown that the position of tubes in the rack during preparation, autoclaving, and incubation may contribute significantly to the error of *L. leichmannii* assay for B_{12}. Randomization of the tubes effectively allows for these factors without seriously increasing the labor involved. A convenient way of applying randomization has been developed. In assays of four unknowns with the

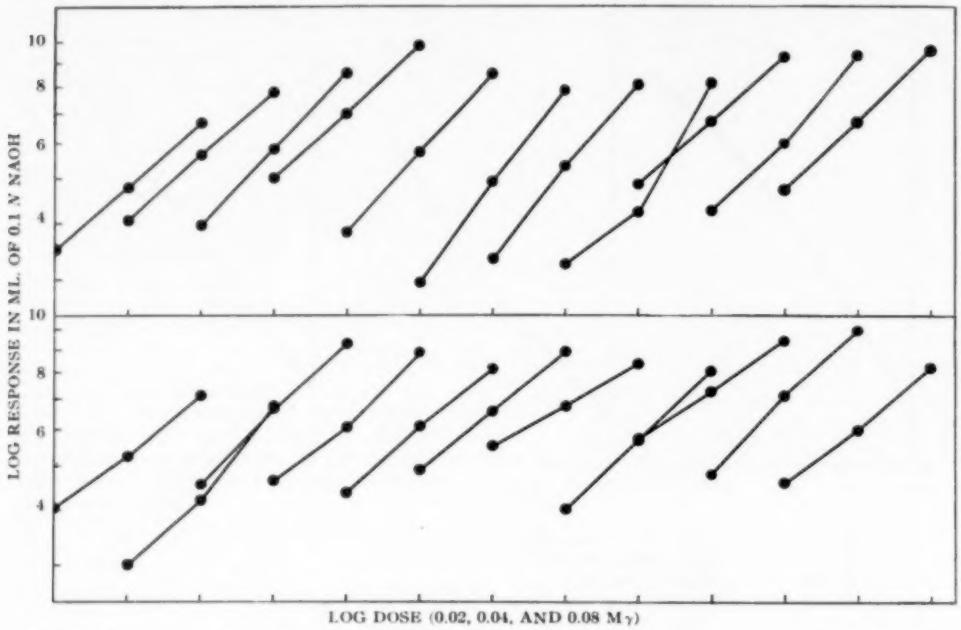

Fig. 2.—Response of *L. leichmanii* to crystalline vitamin B_{12} in consecutive assays by the six-point design.

Upper assays—unrandomized tubes.
Lower assays—randomized tubes.

standard, two tubes are used at each of three levels of the standard and one at each level of the unknowns. These 18 tubes are randomized in a block which may be replicated four times in one rack. If six samples are assayed, three replications of a block of 24 tubes would fit a rack. Each tube is numbered and treated in the usual manner until just before addition of the medium. Tubes are then rearranged in the racks according to the randomized plan which is used until after titration of the acid. The time required to mark the tubes and randomize amounted to only ten to fifteen minutes to set up a rack of 72 tubes. Randomization has been adopted for routine use in this laboratory.

It should be mentioned that while the assay procedure outlined will yield highly reproducible results over a period of months, experience has shown that the assay will occasionally yield an atypical response. The reasons for this behavior are not completely understood, but it has been traced to such factors as inadvertent contamination and atypical growth of cultures. At other times, no reason could be found. While such behavior may constitute a criticism of the method, the fact that such occurrences become rarer with experience tends to reduce the importance of this factor.

Any discussion of simplifying the seventy-two hour titrimetric assay should probably include comments on the merits of the eighteen to twenty-four hour turbidimetric assay. The six-point design has also been applied to the latter test in a limited number of assays. The media was modified by omitting the tomato juice and decreasing the amount of glucose to three-fourths of that recommended in the U. S. P. method. It was found that the response, expressed as the logarithm of the optical density as read on a Coleman Model 11 spectrophotometer, was linear between the limits of 0.02 and 0.16 mγ vitamin B_{12}. Results of assays, using the six-point design at dosage levels of 0.03, 0.06, and 0.12 mγ vitamin B_{12}, indicated that the precision and reproducibility of the turbidimetric assay was of the same order as that indicated above for the titrimetric assay. The single dose method (for unknowns) mentioned above for the titrimetric assay is particularly suited to this rapid procedure.

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A Pharmacological Study of a Homologous Series of Sodium Alkyl Sulfates*,†

By LAURENCE E. GALE‡ and PAUL M. SCOTT

A homologous series of sodium alkyl sulfates, from sodium octyl sulfate to sodium octadecyl sulfate, inclusive, was synthesized and tested pharmacologically. The oral and intraperitoneal LD_{50}'s were determined for each compound. The effect of the applications of each of these sodium alkyl sulfates to nervous tissue and to skeletal, smooth, and cardiac muscles was determined. In each case, sodium dodecyl sulfate (sodium lauryl sulfate) exerted the most profound effect upon each of the selected tissues. These compounds were found to be relatively nontoxic with correspondingly slight effects upon the experimental tissue.

THE PHARMACOLOGY of a homologous series of sodium alkyl sulfates which includes the compounds from sodium octyl sulfate to sodium octadecyl sulfate is the subject of the present investigation. Little pharmacological work has been done with this group of compounds, other than the evaluation of irritant properties and determination of some of the individual toxicities. Emery and Edwards (1) reported that these homologs are less irritating to the human skin than ordinary soap. However, they are more irritating than soap when applied to mucous and ciliated membranes (2). Medicinally, the sodium alkyl sulfates have had considerable use as germicidal detergents in the cleansing and irrigation of wounds (3), and as vehicles in pharmaceutical preparations to aid in the absorption of drugs by way of the skin and mucous membranes (4).

The compound of this series with which much of the work to date has been concerned has been sodium dodecyl sulfate, which, Ginsberg and Robson (4) have shown, increases penetration of sulfacetamide through corneal membranes. The approximate intraperitoneal LD_{50} for sodium dodecyl sulfate in rats has been determined by Epstein, *et al.* (5), to be 0.21 Gm./Kg. Two values for the approximate oral LD_{50} of sodium dodecyl sulfate in rats are listed in the literature: one value is given by Smyth, *et al.* (6), as 2.70 Gm./Kg., the other by Woodard and Calvery (7) as 1.00 Gm./Kg. Additional acute toxicity determinations for sodium dodecyl sulfate or for other sulfates of this series have not been reported.

Klotzbucher (8) states that sodium dodecyl sulfate alters the surface tension and enhances the cell permeability of cardiac muscle. The other members of this series may be of similar

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value as potentiating agents in the administration of therapeutic preparations. However, before such studies are undertaken, the pharmacology of these compounds must be investigated further. Therefore, the purpose of this work has been to study the compounds as individuals and in relation to each other with regard to their toxicities, and to determine their pharmacological effects upon nervous tissue and upon striated, smooth, and cardiac muscle.

EXPERIMENTAL AND RESULTS

Synthesis of Compounds.—For the purpose of a pharmacological investigation of the series, the sodium alkyl sulfates were synthesized to obtain them in as high a state of purity as possible.

Using a modification of an already existing method (9), each of the six sodium alkyl sulfates was synthesized from its corresponding alkyl alcohol[1] by treating the alcohol with a calculated amount of sulfuric acid[2] U. S. P., neutralizing the mixture with sodium hydroxide in a saturated anhydrous alcoholic solution, and filtering rapidly. The filtrate was treated four times with 1-Gm. quantities of activated charcoal[3] and subsequently filtered each time. The clear alcoholic filtrate containing sodium alkyl sulfate was evaporated on a water bath to one-third of its original volume, placed in a refrigerator at 5°, and allowed to remain overnight. The crystals of sodium alkyl sulfate obtained from the refrigerated solution were dried in an oven at 70° and stored over anhydrous calcium chloride in tightly stoppered containers.

The actual yields of sodium alkyl sulfates from this method of synthesis averaged approximately 42% of theoretical. However, analysis of the compounds showed them to be of a high degree of purity. Table I lists the melting points of the pure alcohols as well as the melting points of the alcohols used in this synthesis and shows the analysis data for the sodium alkyl sulfates.

TOXICITY

The animals used in the intraperitoneal and oral toxicity determinations were maintained in a room which was kept at constant temperature (70° F.) and humidity. Tabulations of the number of deaths were taken during a twenty-four hour period following administration. The LD_{50} determinations were carried out according to the method of Dragstedt and Lang (10), elaborated upon by Behrens (11). The numerical results are presented in Table II. A brief description of the symptoms as they occurred by each route of administration is also given in the section on results. Rat and mouse pellets constituted the diet of the experimental animals throughout the toxicity determinations.

Intraperitoneal LD_{50}.—An arbitrary dosage scale was established by preliminary tests, and the 60 mice used in the testing of each compound were divided into subgroups of 10 mice each, five male and five female, each of which was in a weight range of 18–32 Gm. Each of these subgroups received one of the predetermined dosages for the compound being tested. Each animal received a volume of 0.5 cc. of distilled water containing the required dosage of compound per 24 Gm. of body weight. A control group of six mice was used for each group of 60 animals, and each mouse was given 0.5 cc. of distilled water per 24 Gm. of body weight.

Oral LD_{50}.—The oral LD_{50} for each of the six sodium alkyl sulfates was determined using a group of 30 albino rats for each compound tested. The animals were of approximately the same age and weight. Each of the groups of 30 animals were divided into subgroups of five animals. A dosage schedule was established whereby each group of five animals was given, by means of a syringe and oral needle, a dosage which differed by 0.5 Gm./Kg. from each preceding or succeeding dosage. The compounds were administered as aqueous mixtures prepared in such a manner that 1 cc. of detergent preparation was administered for each 100 Gm. of rat. The mixtures administered were warmed to 35°. However, the viscosity of the mixtures of tetradecyl, hexadecyl, and octadecyl compounds at dosages greater than 3.5 Gm./Kg. for the first preparation and 3.0 Gm./Kg. for the latter two preparations was so high that the mixtures could not be forced through an oral needle. Five rats, maintained as controls for each group of 30 animals, were each given 1 cc. of distilled water per 100 Gm. of body weight.

Results of Intraperitoneal and Oral LD_{50} Determinations.—The symptoms observed in the mice receiving intraperitoneal dosages were the same for all compounds that were tested. Initially there was a period of increased activity which was followed by a state of graded depression which eventually resulted in prostration and death. Most of the animals died twelve to eighteen hours after the injections. Autopsy showed the intestines to be swollen and deep red in color. Blood was present in the lumen of the intestine and slight hemorrhages were present in the peritoneal lining. Moribund animals were anesthetized and examined; they showed a severe dilation of the blood vessels of the mesentery and intestine with swelling throughout the visceral area.

The symptoms caused by the oral administration of the compounds were observed to be essentially the same in all rats. These were depression, severe diarrhea which was characterized by the presence of blood, and finally complete prostration followed by death. Autopsy showed varying stages of acute gastritis and congestion of the mucosa of the small intestine. The stomach usually contained some food and was swollen and severely ulcerated. Microscopic investigations of the stomach contents showed the presence of crenated and ruptured red blood cells along with accumulations of cells from the gastric mucosa.

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[1] Eastman Organic Chemicals, Rochester, N. Y. No. 871, *n*-octyl alcohol; No. 370, *n*-decyl alcohol; No. 873, lauryl alcohol; No. 1416, myristyl alcohol; No. 1324, cetyl alcohol; No. 4053, octadecyl alcohol.

[2] A ratio of 1 mole of alcohol to 3 moles of acid was used throughout the syntheses; however, the actual amount of the alcohol used for each synthesis varied from 1–3 Gm.

[3] "Nuchar C," activated charcoal, is manufactured by the West Virginia Pulp and Paper Co., Covington, Va.

TABLE I.—PHYSICAL AND ANALYTICAL DATA

Sodium Alkyl Sulfates	Empirical Formula	M. P. Starting Alcohols, °C.	M. P. Pure Alcohols, °C.[a]	Analyses, %[b] Calcd.	Found
Octyl	$C_8H_{17}SO_4Na$	−15 to −16	−16	C, 41.30 H, 7.31 S, 13.82	C, 41.35 H, 7.33 S, 13.68
Decyl	$C_{10}H_{21}SO_4Na$	6.5–7	7	C, 46.13 H, 8.13 S, 12.31	C, 46.02 H, 8.27 S, 12.14
Dodecyl	$C_{12}H_{25}SO_4Na$	23–24	24	C, 49.97 H, 8.73 S, 11.12	C, 48.27 H, 8.11 S, 10.84
Tetradecyl	$C_{14}H_{29}SO_4Na$	36–39	38	C, 53.13 H, 9.23 S, 10.13	C, 59.30 H, 10.42 S, 7.39
Hexadecyl	$C_{16}H_{33}SO_4Na$	49–50	50	C, 55.78 H, 9.65 S, 9.30	C, 55.97 H, 9.58 S, 9.16
Octadecyl	$C_{18}H_{37}SO_4Na$	56–59.5	58.5	C, 58.03 H, 10.00 S, 8.55	C, 61.60 H, 10.70 S, 7.03

[a] Reported in the literature.
[b] Analyzed by the Clark Microanalytical Laboratory, Urbana, Ill.

TABLE II.—ORAL AND INTRAPERITONEAL LD_{50} RESULTS FOR THE SODIUM ALKYL SULFATES AS DETERMINED FOR MICE AND RATS

Sodium Alkyl Sulfates Tested	Intraperitoneal Injection—pH of Soln.	Intraperitoneal Injection—LD_{50} for Mice, Mg./Kg.	Oral Administration—pH of Soln.	Oral Administration—LD_{50} for Rats, Gm./Kg.
Octyl	6.9	396.0 ± 29	6.8	3.20
Decyl	7.4	284.5 ± 21	6.9	1.95
Dodecyl	7.2	250.0 ± 20	7.6	2.64
Tetradecyl	7.7	342.0 ± 16	...	>3.50
Hexadecyl	8.1	356.0 ± 28	...	>3.00
Octadecyl	8.8	477.0 ± 41	...	>3.00

EFFECT OF THE APPLICATION OF THE SODIUM ALKYL SULFATES UPON NERVE AND MUSCLE TISSUE

Frog's Sciatic Nerve.—The frog sciatic nerve preparation was used to determine the effects of the sodium alkyl sulfates upon nerve conduction. Two frogs were used for each compound. Both sciatic nerves and both gastrocnemius muscles of each pithed frog were isolated *in situ*. Small paraffin cups were placed under each nerve. These cups were filled with the dilution of the compound to be tested and the one under the left sciatic was filled with normal amphibian saline. The nerves were stimulated simultaneously at five-second intervals with a threshold stimulus until the muscles were fatigued. The current was applied by way of platinum electrodes and a Harvard inductorium. The current source was a storage battery. The following dilutions of the sodium alkyl sulfate being tested was applied to each nerve preparation: 1:100,000, 1:50,000, 1:20,000, 1:5,000, and 1:100.

Striated Muscle.—A saline dilution of 1:100 for each of the six sodium alkyl sulfates was applied to the right gastrocnemius of 36 frogs. The muscles

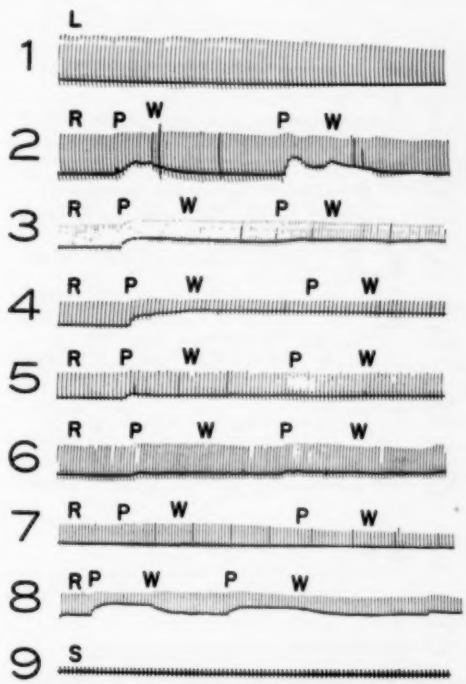

Fig. 1.—Reactions of frog gastrocnemius to direct application of 1:100 dilutions of the six sodium alkyl sulfates tested and the gastrocnemius response to a 1:100 dilution of potassium chloride. 1—control; 2—sodium octyl sulfate; 3—sodium decyl sulfate; 4—sodium dodecyl sulfate; 5—sodium tetradecyl sulfate; 6—sodium hexadecyl sulfate; 7—sodium octadecyl sulfate; 8—potassium chloride; 9—signal magnet recording five second stimulations. L = left gastrocnemius; R = right gastrocnemius (test); P = point of application; W = point of application of saline wash; S = signal magnet.

were stimulated through the sciatic nerve at five-second intervals and the left gastrocnemius of each frog was used as a control. Uniform applications were made directly to the muscle with a medicine dropper. The control muscle was bathed constantly with amphibian saline. In this set of experiments, each test muscle was exposed to two separate applications of the same dilution with saline washings before and after each application.

Six additional frog gastrocnemius muscles were exposed to 1:100 dilutions of potassium chloride to compare the action of an excess of potassium ions with the responses elicited by the sodium alkyl sulfates.

Results of Sciatic Nerve and Striated Muscle Experiments.—The dilutions of the compounds ranging from 1:100,000 to 1:100 had no effect upon the normal conductivity of the sciatic nerve in the frog.

Dilutions of the sodium alkyl sulfates applied to the gastrocnemius muscle of the frog caused an elevation of the base line of successive contractions but did not decrease the threshold of response. All responses were of an irreversible nature except those effected by sodium octyl sulfate which were readily reversible. Figure 1 shows the comparative effects of the applications of the six compounds to gastrocnemius muscle as well as the response of gastrocnemius muscle to a 1:100 dilution of potassium chloride under the same conditions.

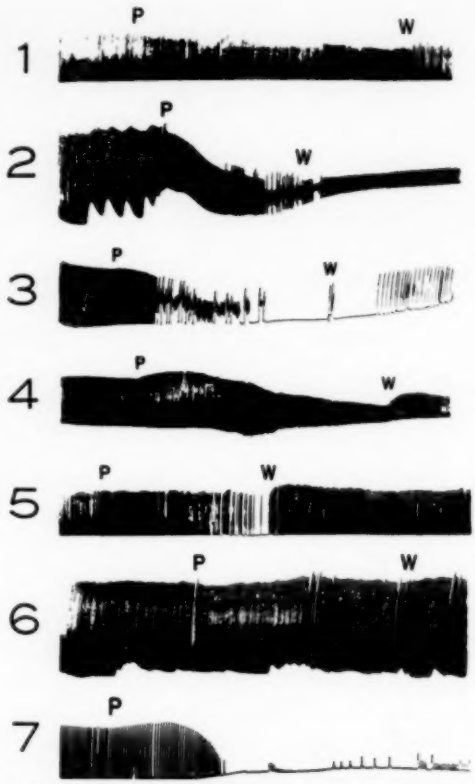

Fig. 3.—Responses of isolated turtle heart to 1:1,000 dilutions of each of the six sodium alkyl sulfates, and a 1:100 dilution of potassium chloride. 1—sodium octyl sulfate; 2—sodium decyl sulfate; 3—sodium dodecyl sulfate; 4—sodium tetradecyl sulfate; 5—sodium hexadecyl sulfate; 6—sodium octadecyl sulfate; 7—potassium chloride. P = point of introduction of detergent solution; W = point of introduction of Ringer's solution wash.

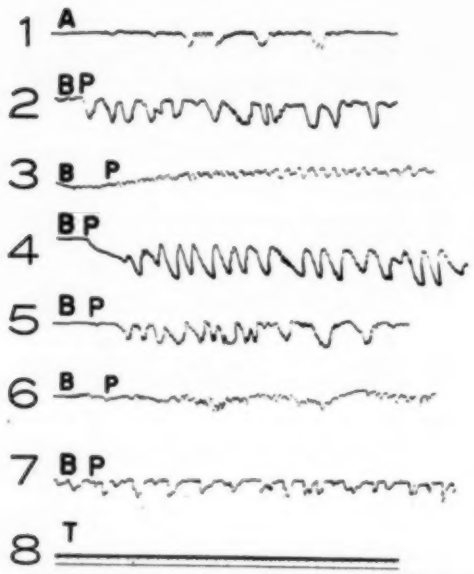

Fig. 2.—Rat intestinal responses to dilutions of each of the six sodium alkyl sulfates in Ringer's Solution. 1—Control; 2—1:1,000 dilution of sodium octyl sulfate; 3—1:10,000 dilution of sodium decyl sulfate; 4—1:20,000 dilution of sodium dodecyl sulfate; 5—1:5,000 dilution of sodium tetradecyl sulfate; 6—1:500 dilution of sodium hexadecyl sulfate; 7—1:100 dilution of sodium octadecyl sulfate; 8—timing record. A = normal contraction, B = test responses, P = point of administration, T = timing record in minutes and seconds. The distance between the horizontal lines in the original kymogram was 4 mm.

Smooth Muscle.—In an attempt to duplicate as closely as possible the smooth muscle activity which would occur in normal animals after the oral administration of the sodium alkyl sulfates, a modification of a method designed by Gomes da Costa (12) was utilized. The original method was designed to utilize guinea-pig intestine, but by making slight modifications in the technique, white rats could be used throughout this smooth muscle work.

A total of 30 rats were used in this test. Each compound was tested on five rats and was administered in the following dilutions in the order listed: 1:100,000, 1:80,000, 1:60,000, 1:40,000, 1:20,000, 1:10,000, 1:5,000, 1:1,000, 1:500, and 1:100. The compounds were introduced into the intestine dis-

solved in warm Ringer's solution, U. S. P., and the intestinal activity was recorded over a five-minute time interval. Following each administration the intestinal segment was thoroughly washed with the Ringer's solution.

Results of Smooth Muscle Tests.—The concentration of the sodium alkyl sulfates necessary to produce an alteration in the intestinal activity of albino rats varied with the particular member of the series; however, the intestinal responses after the administration of each compound were qualitatively the same. The concentrations of each compound necessary to bring about a uniform increase in intestinal activity are as follows: sodium octyl sulfate, 1:1,000; sodium decyl sulfate, 1:10,000; sodium dodecyl sulfate, 1:20,000; sodium tetradecyl sulfate, 1:5,000; sodium hexadecyl sulfate, 1:500; and sodium octadecyl sulfate, 1:100.

Figure 2 shows kymograms of the rat intestinal response to the preceding dilutions of each compound.

Cardiac Muscle.—Tests to ascertain the influence of the sodium alkyl sulfates upon cardiac muscle were performed upon the isolated hearts of 30 turtles. Five turtles were subjected to 1:1,000 dilutions of each of the six compounds tested. All of the dilutions were made using amphibian Ringer's solution with 0.1% dextrose added. The Ringer's solution with dextrose was also used as a wash and as a control. Each heart was constantly moistened externally by a controlled flow of the same solution.

Potassium chloride (1% solution) was also perfused through the isolated hearts of six other turtles and the responses were recorded in order to compare the action with that of the sodium alkyl sulfates.

Results of Cardiac Musculature Tests.—The dilutions of sodium octyl and octadecyl sulfates produced no significant effects upon cardiac activity, but sodium decyl, dodecyl, tetradecyl, and hexadecyl sulfates produced a graded decrease in amplitude, output, and frequency. Figure 3 shows the kymograms obtained as the result of perfusing the isolated turtle heart with 1:1,000 dilutions of the sodium alkyl sulfates. It also shows the response of the isolated turtle heart to 1:100 dilutions of potassium chloride, using the same procedure.

SUMMARY

1. A modified method has been devised for preparing the following pure sodium alkyl sulfates: sodium octyl sulfate, sodium decyl sulfate, sodium dodecyl sulfate, sodium tetradecyl sulfate, sodium hexadecyl sulfate, and sodium octadecyl sulfate.

2. The intraperitoneal and oral LD_{50} was established for each of the six sodium alkyl sulfates in mice and rats, respectively. With the exception of the decyl compound given orally, sodium dodecyl sulfate is the most toxic of the group.

3. The sodium alkyl sulfates did not alter conduction in the sciatic nerves of the frogs.

4. The sodium alkyl sulfates altered the normal activity of frog striated muscle, producing (with the exception of sodium octyl sulfate) an irreversible potassium-like action on the contractility of the muscle. They elicited an increase in activity in the rat intestinal smooth musculature and caused a decrease in frequency and amplitude of the turtle-heart.

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Rubber Closures for Containers of Parenteral Solutions*

I. The Effect of Temperature and *p*H on the Rate of Leaching of Zinc Salts from Rubber Closures in Contact with (Acid) Solutions

By SOLOMON REZNEK

Among substances which appear on the surface of molded rubber closures used for parenteral solutions are zinc salts added to assist the curing process. Although the zinc initially present on the surface may be removed by washing, it occurs in the solutions in contact with the rubber, either by migration of additional quantities of zinc from the interior of the rubber or by penetration of the solution into the rubber. The dissolved zinc may reach a level which may be physiologically objectionable or may be the cause of insoluble particle formation by interaction with other constituents of the solution or container. The effect of temperature and *p*H on the rate of leaching of zinc salts is investigated.

RUBBER is seldom cured (vulcanized) by the use of sulfur alone. This simple procedure is not only uneconomically slow but forms rubber of inferior quality by modern standards. Modern curing techniques, almost without exception, use vulcanization "accelerators," which in turn require accelerator "activators," usually metal oxides, of which zinc oxide is the most common.

The mechanism of the curing reaction between rubber and sulfur or between rubber, sulfur, accelerator, zinc, etc., has not yet been completely clarified, although research on the subject continues. There is seldom, if ever, a 100% reaction in the sense that all of the original ingredients are consumed in the formation of a single "rubber" entity. The incompleteness of the reaction is demonstrated in part by the phenomenon of "bloom," which consists of that portion of the ingredients which have migrated to the surface of the cured rubber.

Almost any of the ingredients or their decomposition products, except free rubber hydrocarbon, may be demonstrated in the bloom. In a common rubber formula, these include the "active" ingredients: sulfur, accelerators (and their decomposition products), and zinc. In addition, there are also the inert fillers, such as wax and blanc fixe (barium sulfate), and other relatively inert ingredients, e. g., fatty acid (usually stearic), softening agents (oils), coloring agents, etc.

Some types of bloom are desirable, e. g., waxes may retard oxidation or improve mechanical action of movable parts. In general, however, excessive blooms are to be avoided. Experience has shown how to control the more common types of bloom, such as those due to excess sulfur, wax, etc., but little work seems to have been done to control blooming of trace amounts of other rubber constituents. The problem is also complicated by constituents which may bloom relatively little in air, but to a different degree when the rubber surface is in contact with a solution, owing to continuous removal of the constituents by the solution.

It is probable that blooming has not been investigated more extensively partly because of a lack of satisfactory analytical methods and partly because the significance of some trace constituents of the bloom has not been fully realized. However, there is evidence that some of the ingredients normally present in rubber may have a greater effect than had previously been thought possible and the realization of this effect has stimulated the present work.

Of the normal constituents of so-called "biological" rubber (a term denoting rubber closures for injectable pharmaceuticals), only zinc and the accelerators have sufficient solubility or chemical reactivity to warrant being seriously considered for either their physiological effect or their effect on solution stability. (Several of the other constituents may be objectionable, if only for contributing insoluble particles to the solution; zinc salts may also contribute indirectly to particle development by the formation of insoluble complexes with the solution ingredients.) The present paper deals with the leaching behavior of zinc from molded rubber stocks in contact with certain solutions. The rubber stocks dealt with in this paper are somewhat specialized in that they contain paraffin wax purposely added in excess of the proportion in which the wax is soluble in the rubber. The data obtained from this

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paper should not be quantitatively extrapolated to rubbers of other types, composition, or cured under different conditions.

MATERIALS TESTED

Anesthetic Solutions in Cartridges.—Since this laboratory has been primarily concerned with dental anesthetic cartridges, preliminary data were gathered on solutions contained in such cartridges, and the effects of time and temperature of storage on the zinc content of these cartridges were investigated.

The solutions tested in cartridges are typical of dental anesthetics and contain the following: (*a*) an anesthetic such as procaine or a procaine analog; (*b*) a very small proportion of a vasoconstrictor (e. g., epinephrine); (*c*) inorganic salts to make the solution isotonic; and (*d*) an antioxidant, such as bisulfite. The *p*H of these solutions when compounded is between 4.0 and 5.0 but may drop below 4.0 on standing, largely owing to oxidation of the sulfite.

Closures (Plungers) for Cartridges.—The cartridges referred to above were fitted with two closures, a movable closure or "plunger" and a fixed closure or "diaphragm." Although the information gathered from such filled cartridges is useful, interpretation of the data is complicated by the presence of two types of closures in each cartridge. The remainder of the work was therefore devoted to a study of the leaching behavior of one type, i. e., "plungers."

Plungers from two sources of supply were treated. The formulas used by the two manufacturers of these plungers were quite similar; the ingredients were used in essentially the proportions shown in Table I. (A plunger formula characteristically contains a considerable proportion of paraffin wax, included to improve the working of the plunger in the cartridge so that it will move readily when acted on by the piston of the syringe).

TABLE I.—TYPICAL RUBBER FORMULA FOR CARTRIDGE PLUNGERS

	Parts	Per Cent of Total
Pale crepe rubber	100	40.6
Blanc fixe (pptd. barium sulfate)	125	50.6
Zinc oxide	4.5	1.8
Stearic acid	2	0.8
Sulfur	2	0.8
Accelerator(s) (mercaptobenzothiazole and thiuram monosulfide types)	1 (or less)	0.4
Paraffin wax (m. p. 53–55°)	10	4.1
Titanium dioxide	2	0.8

Before the plungers were subjected to the leaching solution, they were first washed by a procedure commonly used in the ordinary factory processes, consisting of a wash with warm (70°) detergent (alkaline metaphosphate type), followed by two rinses with warm distilled water. The purpose of this procedure was to remove the surface bloom; even though this bloom may contain a portion of the zinc salts, among other things, it appeared desirable to simulate ordinary washing practice.

DETERMINATION OF SOLUBLE ZINC

The quantities of zinc which were found to have leached from the ordinary stocks were of the order of μg. per cc. of leaching solution. The method which appeared to be best adapted for the rapid routine determination of a number of samples containing extremely minute quantities of zinc is one which depends upon the fluorescence of the precipitate formed by the action of 8-hydroxyquinoline on zinc salts. The method was adapted from that of Merritt (1) by M. E. Auerbach of the Sterling-Winthrop Research Institute, Rensselaer, New York, and was further modified by this laboratory.

Reagents.—The following reagents were required:

(*a*) Ammonium acetate solution. Dissolve 15.4 Gm. of crystals, C. P., in water and dilute to 100 cc. Adjust the *p*H with ammonia, if necessary, to 7.0 ± 0.2.

(*b*) Gum acacia solution. Dissolve 2 Gm. in 100 cc. of water; filter.

(*c*) 8-Hydroxyquinoline solution. Dissolve 0.25 Gm. in 5 cc. of glacial acetic acid; dilute to 50 cc. with water.

(*d*) Blank anesthetic solution, prepared and kept in Pyrex glass-stoppered ware.

Prepare reagents (*a*) and (*c*) frequently, preferably daily.[1]

Method.—Pipette 1.0-cc. samples (less if the solution contains more than 25 μg. of zinc per cc.) into fluorometer tubes. Adjust the volume to exactly 2.0 cc. with a blank solution and then add 1.5 cc. of distilled water, 1.0 cc. of the ammonium acetate solution, and 0.5 cc. of the acacia solution. Mix, add 0.25 cc. of the hydroxyquinoline solution, and mix again. Shake each tube thoroughly and then allow to stand for at least one-half hour, shaking two or three times during the interval. Add 5.0 cc. of distilled water, mix, and read in the fluorometer.

Together with the samples, prepare a set of standards containing 5, 10, 15, 20, and 25 μg. of zinc (these are conveniently prepared from a standard containing 25 μg. of zinc per cc.). Dilute to 1.5 cc. with water and add 2.0 cc. of blank solution. Add 1.0 cc. of ammonium acetate, and 0.5 cc. of acacia; mix, add the 0.25 cc. of hydroxyquinoline solution, and proceed as for the samples. At the end of the half-hour period of standing, add 5.0 cc. of distilled water, mix again thoroughly, and read the solutions in a fluorometer. In this work, a Coleman photofluorometer was employed; the B–1 was used as the primary filter and the PC–2 as the secondary filter. Set the blank at zero and construct a curve from the standards. For interferences consult the original procedure (1).

When dilute acid solutions, the *p*H of which was generally lower than the anesthetic solutions, were used as leaching agents, the quantity of acetate

[1] In this part of the work it was convenient to use anesthetic solution as a blank to establish the same conditions between standards and sample solutions. Other solutions may be used so long as they do not introduce interfering salts or adversely affect the *p*H.

buffer was increased to 2.5 cc. and the volumes were adjusted with distilled water instead of anesthetic solution. In each case, the standards were prepared to correspond to the sample solutions.

Since the method depends on the fluorescence of a precipitate whose characteristics must be exactly reproduced, it is sensitive to experimental conditions; therefore it is necessary to establish a precise and consistent technique of adding reagents, measuring volumes, etc. Experience has shown that the zinc can be determined within 1 μg., a precision equivalent to approximately ±5% at the upper end of the range (i. e., around 25 μg.).

Method of Calculation.—The zinc content is expressed either as parts per million (p. p. m.) or as milligrams per square inch of rubber surface The first expression is useful when solutions are to be compared for physiological effects arising from their zinc content. The latter form allows a more direct expression of the function of the surface area in furnishing soluble zinc to the solution with which it is in contact. In addition, comparison may be made more readily between different sizes and shapes of rubber closures, in so far as their surface areas can be calculated. Comparison is especially facilitated in work with experimental rubber formulas, where it is more convenient to use flat slabs of rubber rather than specially molded closures. Sections of the slabs molded for the standard tensile specimens according to the A. S. T. M. (2) are convenient for this purpose and also allow a comparison of tensile data with zinc leaching data.

P. p. m. are calculated by direct comparison with the standards, allowing for any differences in sample size.

Milligrams per square inch are calculated from the p. p. m. content of the solution by the following formulas:

CLOSURES (PLUNGERS)—WHOLE SURFACE EXPOSED AS IN TABLES III AND IV

(15 closures–5.1 sq. in.–per 50 cc. of leaching solution)

$$\text{Mg./sq. in.} = \frac{\text{P. p. m.} \times \frac{50}{1{,}000}}{5.1} = \text{P. p. m.} \times 0.01$$

CLOSURES IN CARTRIDGES—ENDS ONLY EXPOSED[1]

(2 closures, each of approximate end area 0.06 sq. in., per 2.5 cc. of solution)

$$\text{Mg./sq. in.} = \frac{\text{P. p. m.} \times \frac{2.5}{1{,}000}}{0.06 \times 2} = \text{P. p. m.} \times 0.02$$

DISCUSSION

Dental Anesthetic Solution in Cartridges

Effect of Temperature of Storage on Rate of Zinc Leaching.—The data on the zinc content of solutions in cartridges kept for different lengths of time under varying temperature conditions are shown in Table II. It will be noted that, although the cartridges of item (2) were fitted with plungers containing 18% of zinc oxide [nine times as much as the plungers of items (1) and (3)], they did not exhibit significantly higher zinc levels than the other cartridges until after approximately four months of storage at room temperature (R. T.). At 45°, a significant difference appeared within two days; this temperature is frequently approached under some storage conditions, such as on shipboard or in the tropics.

The data in Table II indicate that some degree of accelerated aging is desirable, since the rate of leaching at ordinary room temperatures is such that little distinction was made even between radically different formulas until the test was run for a period extending into several months. On the other hand, a too-greatly accelerated aging should be prevented since it may yield results entirely unrelated to those obtained by ordinary aging conditions.

TABLE II.—ZINC CONTENT OF ANESTHETIC CARTRIDGES—COMPARISON OF EFFECT OF STORAGE TEMPERATURE ON DIFFERENT LOTS OF CLOSURES

Age	P. p. m. of Zinc[a]							
	2 Days		14 Days		1 Mo.		4 Mo.	8 Mo.
Storage temperature, °C.	R. T.	45°	R. T.	45°	R. T.	45°	R. T.	R. T.
Closure Combination								
(1) Mfgr. A[b] 1.6% ZnO[c] plunger 2.5% ZnO diaphragm	3	11	4	15	5	19	9	8
(2) Mfgr. A 18% ZnO[c] plunger 2.5% ZnO diaphragm	4	33	3	44	7	69	11	12
(3) Mfgr. B[b] 2.0% ZnO[c] plunger 1.4% ZnO diaphragm	7	13	4	20	9	27	10	8

[a] Each figure is an average of 9 individual cartridges; p. p. m. of Zn in solution from cartridge containing an average of 2.5 cc.
[b] "Mfgr. A" and "Mfgr. B" represent different closure sources; zinc values are from experimental cartridges stoppered with combinations of closures as shown and filled with anesthetic solution.
[c] Per cent ZnO on basis of total mix.

Plungers for Dental Cartridges

Effect of pH and Composition of Leaching Solution on Zinc Leaching Rate.—In Tables III and IV, the zinc leaching rates at 50° in four solutions varying in *p*H from 1.1 to 4.0 are compared. The

[1] This calculation is approximate and is for comparative purposes only; one end of the cartridge is fitted with a closure (diaphragm) of different shape whose contact surface is more complex than that of the symmetrically shaped plunger.

TABLE III.—ZINC LEACHING RATES AT 50° IN ANESTHETIC SOLUTION (*p*H 4.0) COMPARED TO 0.01 *N* HCl (*p*H 2.0)

	Mg. Zn Leached/Sq. In. Closure Surface			
	Lot 1		Lot 2	
Days	Anes. Soln., *p*H 4.0	0.01 *N* HCl, *p*H 2.0	Anes. Soln., *p*H 4.0	0.01 *N* HCl, *p*H 2.0
2	0.24	0.32	0.19	0.17
4	0.30	0.40	0.23	0.40
6	0.33	0.42	0.27	0.43
11	0.49	0.50	0.32	0.49
15	0.57	0.63	0.35	0.59
25	0.61	0.69	0.37	0.63
32	0.62	0.73	0.37	0.65
62	0.64	0.78	0.39	0.70

TABLE IV.—ZINC LEACHING RATES AT 40° IN 0.005 *N* HCl (*p*H 2.4) COMPARED TO 0.1 *N* HCl (*p*H 1.1)

0.01 *N* HCl (pH 2.0)

	Mg. Zn Leached/Sq. In. Closure Surface			
	Lot 3		Lot 4	
Days	0.005 *N* HCl	0.1 *N* HCl	0.005 *N* HCl	0.1 *N* HCl
2	0.34	0.37	0.29	0.38
4	0.62	0.62	0.70	0.75
10	0.68	0.71	0.91	0.81
14	0.68	0.71	0.93	0.84
21	0.68	0.74	0.96	0.89
28	0.68	0.81	1.03	0.93
35	0.68	0.87	1.03	0.96
42	0.71	0.87	1.08	...
56	0.74	0.90	1.10	1.16

latter consisted of an anesthetic solution, commonly in contact with these closures, which has a considerable degree of buffering capacity. The other three solutions consisted of dilute, unbuffered hydrochloric acid. All represented more acid conditions than are ordinarily encountered in injectable preparations and would be expected to exert at least as great a degree of leaching action as any of the common injectables.

Three observations may be made upon these data:

(1) As in the case of the filled cartridges (Table II), the quantity of zinc leached tends to reach a maximum after a relatively short interval. This period depends, however, on the storage temperature and to some degree on the acidity.

(2) Within the range of acidities studied (which varied 20-fold in the case of the unbuffered solutions), the acid strength had less effect on the leaching rate within a particular lot than on the variations among lots.

(3) The maximum zinc leached when stable conditions were achieved was a small proportion of the total zinc content of the closure. For example, the maximum value in the tables (Table IV, 1.16 mg. per sq. in.) represented 17% of the total zinc content. Subsequent experiments confirmed the observation that only a small proportion of the total zinc content of the closures is leached under ordinary storage conditions.

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Rubber Closures for Containers of Parenteral Solutions*

II. The Relation between Zinc Content of Cured Rubber and Acid-Soluble Zinc

By SOLOMON REZNEK†

The relation between the total zinc content and zinc extracted by dilute hydrochloric acid at 50° and 70° is examined. The extractable zinc approaches a constant level which is a fraction of the total zinc content of the rubber. Evidence is presented that at zinc oxide levels below 1.5 parts per hundred parts of rubber the extractable zinc tends to reach a minimum.

IN ADDITION to functioning as an accelerator-activator, zinc oxide is used as a stiffener in amounts up to 50 per cent or more and is considered a good material for this purpose. As an activator, however, relatively small proportions are used; it is generally stated (1) that between one and five parts of zinc oxide per 100 parts of crude rubber is needed to activate the commonly used accelerators for molded goods. In ordinary usage it is not necessary and not the practice to

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determine the minimum zinc requirement precisely. The closures dealt with in Part I of this series are quite typical in containing four to five parts of zinc oxide per 100 parts of rubber (which amounts to approximately 2 per cent on the total mix basis, since the rubber content was 40 per cent of the mix).

With the object of reducing dissolved zinc to a minimum, it was decided to investigate the relationship between the soluble (leachable) zinc and the total zinc content. The data in Table II (Part I) suggest that the relationship is not necessarily a direct one and that there may be factors which affect the leachable zinc out of proportion to the increase in total zinc.

Speculation on the factors affecting the potential solubility of the zinc in or from the vulcanized rubber is connected with the general problem of the structure of vulcanized rubber, which is still being actively investigated. The complexity of the problem is suggested by considering that at least a portion of the zinc in cured rubber may be in one or more of the following states: (*a*) The unchanged oxide; (*b*) the sulfide formed by reaction with sulfur; (*c*) a fatty acid salt, e. g., the stearate; (*d*) a salt of an accelerator or an accelerator decomposition product; (*e*) bound in the structure of the rubber hydrocarbon, either directly or through sulfur or accelerator linkages.

With so many possibilities, it is difficult even to describe the migration mechanism forming surface "bloom," which is probably related to the amount of dissolved zinc. Moreover, solution of the zinc does not necessarily depend only on such zinc as has migrated to or near the surface. Diffusion of the solvent into the rubber or (dissolved) zinc salts out of it must also be considered; on the other hand, diffusion is dependent on the characteristics of rubber as a semipermeable membrane.

Without attempting to determine the specific state of the zinc, it was hoped that evidence could at least be gathered to show whether zinc in cured rubber, when used in its ordinary proportions as an activator, exists (1) entirely in a free form, i. e., readily leached by mild solvents such as dilute acids which are capable of dissolving zinc but which do not affect the rubber structure; (2) entirely bound, i. e., not readily leached by such solvents; or (3) partly bound, i. e., partly as excess or "free" zinc. If (1) is the case, then the leachable zinc should be in direct proportion to the total zinc content. That is to say, given sufficient time, all the zinc would be dissolved from the rubber structure. In (2), no leachable zinc

TABLE I.—ZINC LEACHED FROM RUBBER BY 0.1 *N* HCl, *p*H 1.2[a, b]

Stock No.	Zn Oxide Content P. H. R.[d]	Zn Oxide Content %[e]	Time of Cure, Min.	Breaking Tensile[f]	P. P. M. at 50° C.[c] 2 Days	7 Days	14 Days	28 Days	42 Days	56 Days
1	1.00	0.41	4	2945	9	10	11	13	13	14
			10	2205	5	6	7	8	10	10
2	1.25	0.51	4	3096	11	14	16	23	23	23
			10	2036	6	7	8	10	10	11
3	1.50	0.61	4	3015	15	22	24	30	33	31
			10	2306	10	13	18	21	24	26
4	1.75	0.71	4	2525	14	24	28	28	33	31
			10	2370	11	14	15	18	21	24
5	2.00	0.81	4	2933	19	35	42	58	62	64
			10	2610	15	29	36	64	66	68
6	2.50	1.01	4	2993	23	41	44	64	72	72
			10	2593	19	35	40	64	76	78
					P. P. M. at 70° C.[c]					
1	1.00	0.41	4	2945	10	20	20	20	20	19
			10	2205	6	20	20	20	24	20
2	1.25	0.51	4	3096	15	20	20	22	22	19
			10	2036	5	22	24	22	22	19
3	1.50	0.61	4	3015	16	24	28	30	36	34
			10	2306	8	20	24	28	36	32
4	1.75	0.71	4	2525	34	58	73	75	78	80
			10	2370	22	43	70	68	68	70
5	2.00	0.81	4	2933	34	63	100	108	108	105
			10	2610	28	43	83	90	93	93
6	2.50	1.01	4	2993	38	120	135	135	140	140
			10	2593	42	105	125	135	145	150

[a] Each figure is average of values from duplicate test piece.
[b] 0.5 x 2.0 x 0.08-in. sections (2.5 sq. in. total surface) in 50 cc. of leaching solution—av. wt., 2.0 ± 0.2 Gm.
[c] P. P. M. Zn in 50 cc. of leaching solution.
[d] P. H. R.—Parts ZnO per 100 parts crepe rubber.
[e] Per cent ZnO in mixed rubber stock.
[f] Tensile in lb./sq. in.

TABLE II.—MG. ZINC LEACHED PER SQUARE INCH OF RUBBER SURFACE[a] IN CONTACT WITH 0.1 *N* HCl[b]

Stock No.	Zn Oxide Content P. H. R.[c]	Zn Oxide Content %	ZnO (as Zn) in Test Pieces,[d] Mg.	50°C. 2 Days	50°C. 7 Days	50°C. 14 Days	50°C. 28 Days	50°C. 42 Days	50°C. 56 Days	50°C. % of Total
1	1.00	0.41	6.6	0.14	0.16	0.18	0.22	0.24	0.24	9.1[e]
2	1.25	0.51	8.2	0.18	0.22	0.24	0.34	0.34	0.34	10.4
3	1.50	0.61	9.9	0.26	0.36	0.42	0.52	0.58	0.58	14.7
4	1.75	0.71	11.5	0.26	0.38	0.44	0.46	0.54	0.56	12.2
5	2.00	0.81	13.2	0.34	0.64	0.78	1.22	1.28	1.32	25.0
6	2.50	1.01	16.5	0.42	0.76	0.84	1.28	1.48	1.50	22.8
							70°C.			
1	1.00	0.41	6.6	0.16	0.40	0.40	0.40	0.44	0.40	15.2
2	1.25	0.51	8.2	0.20	0.42	0.44	0.44	0.44	0.38	11.6
3	1.50	0.61	9.9	0.24	0.44	0.52	0.58	0.72	0.62	15.2
4	1.75	0.71	11.5	0.56	1.02	1.44	1.44	1.46	1.50	32.6
5	2.00	0.81	13.2	0.62	1.06	1.84	1.98	2.02	1.98	37.5
6	2.50	1.01	16.5	0.80	2.26	2.60	2.70	2.86	2.90	43.8

[a] Mg./sq. in. $= \frac{\text{P. P. M. (Av. of 2)} \times (50/1000)}{2.5} = \text{P. P. M.} \times 0.02$

[b] Each figure av. of 4 determinations, i. e., 2 cures, each in duplicate.

[c] P. H. R.—Parts ZnO per 100 parts crepe rubber.

[d] Test pieces of 2.5 sq. in. area weighing 2.0 Gm.

[e] Per cent of total zinc content leached from stock in 56 days: e. g.,

$$\frac{(0.24 \text{ mg./sq. in.})(2.5)(100)}{6.6} = 9.1\%$$

would be found, and in (3) the leachable zinc would be proportional only to the unbound fraction. (A possibility also exists that there is a difference between the surface and interior of the rubber structure so that some forms of zinc would be dissolved from the surface but would not be accessible in the interior, because of the semi-permeable nature of the rubber membrane.)

MATERIALS TESTED

In contrast to the closures tested as described in Part I, the materials in the present test consisted of sections of test slabs of which other portions had been used for tensile tests. The standard dumb-bell shaped tensile test specimens are cut from these slabs, which are flat pieces of rubber approximately 6 x 6 x 0.08 in., molded by a standard procedure (2). Sufficient material remains after cutting out several tensile specimens to provide a number of 0.5 x 2.0-in. sections for the leaching tests. Sections of this size are of convenient dimensions to allow placement in 50-cc. bottles in a nearly upright position so that practically all surfaces are in contact with leaching solution.

The composition of the rubber stocks[1] was very similar to that given in Table I (Part I), except for a change in accelerators and a range in zinc content from 1.0 to 2.5 parts. Preliminary work was done with stocks[1] whose zinc content varied from 0 to 25 parts. The results indicated that the zinc content required for a good cure was somewhere below 2.0 parts and the series was then prepared with the object of determining the zinc requirement more critically. The tensile values given in Table I were determined by the Monsanto Laboratory, Nitro, W. Va.

[1] Provided through the courtesy of K. E. Creed, Jr., of the Rubber Service Department, Monsanto Chemical Co., Nitro, W. Va.

Method.—As noted above, 0.5 x 2.0 x 0.08-in. sections were cut out of the slabs which had been used for tensile determinations. The sections were washed and rinsed in the same manner as the plungers (see Part I), placed in bottles with vinylite-lined screw caps, and covered with 50 cc. of 0.1 *N* HCl. Each test was run in duplicate, i. e., two sections were cut from each slab and each section was independently leached. The values in Table I are the averages of these duplicate tests.

The bottles were kept at the indicated temperatures (50° and 70°) and samples were withdrawn at the intervals shown. The contents were not agitated, except for occasional mixing by inverting the bottle. The sample sizes were decreased to as low as 0.10 cc. as the zinc concentration increased.

The method of analysis was as described in Part I with the modification of buffer content and base solutions as indicated. Owing to the sampling error, the values in the higher ranges are estimated to be subjected to an approximate variation of $\pm 10\%$.

DISCUSSION

It is indicated from the data of Tables I and II that the zinc content of molded rubber stocks may be varied within a range of approximately 1.0 to 1.5 parts without appreciably affecting either the tensile strength or the amount of zinc leached from the stock by dilute acid at 50° to 70°. [Zinc oxide content is stated as P. H. R. (parts per 100 parts of crude rubber) as well as in terms of per cent of the total mix. The first mode of expression (P. H. R.) is usual in rubber technology and is well fitted to the uses of the rubber compounder, since the properties of vulcanized rubber depend largely on the individual ratio of each of the ingredients to the crude rubber content. On the other hand, expression in terms of percentage of the total (finished) mix is more useful to the analyst of the finished product.] Increase in the zinc content beyond 1.5 parts does not significantly affect the

tensile values but may greatly increase the soluble zinc. This "zinc break-point," as it may be described, varies somewhat with leaching temperature, and may also vary with the type, composition, and condition of cure of the rubber. If its existence is confirmed for a sufficient series of stocks, it will be of practical importance for the rubber user interested in the minimum-leaching stock, and possibly of theoretical interest in distinguishing between zinc involved in the curing reaction and that acting only as filler.

The data of Table I indicate a secondary point of some interest. It will be noted that the ten-minute cures, in general, exhibited less soluble zinc than the four-minute cures, whereas the tensile strengths were in the reverse order. (Even though tensile determinations at best are subject to a 5% error, the consistency of the values appears to be significant). The tensile data indicate that the ten minute cures represent overcuring rather than undercuring. However, while overcuring may tend to bind the zinc more tightly, it is questionable whether the practice would be desirable in view of the fact that overcured stocks are generally less stable in regard to aging.

In Table II, the values for the four-minute and ten-minute cures of Table I were combined and recalculated in terms of milligrams of zinc leached per square inch of rubber surface. In addition, the proportion of the total zinc content which was leached when stable conditions were reached (eight weeks or less) is shown. It will be seen that, except for the relatively high-zinc stocks at the somewhat higher temperature, only a minor proportion of the total zinc is removed under the leaching conditions used.

Such behavior of the zinc was noted previously with closures alone and with closures in cartridges, as described in Part I. It is difficult to determine whether the soluble portion of the zinc simply represents that fraction of the total zinc present in the surface layer or whether leaching extracts zinc present in certain soluble forms throughout the body of the rubber. The occurrence of a "break-point" tends to support the latter hypothesis, since it indicates that a certain proportion of the zinc is always in an insoluble form, i. e., insoluble under the described condition.

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Antibiotic Activity of Seaweed Extracts. II. *Rhodomela larix*[*,†]

By HENRY G. MAUTNER, GRACE M. GARDNER, and ROBERTSON PRATT

Ether extracts of the marine red alga, *Rhodomela larix*, inhibit the growth of several species of Gram-positive and Gram-negative pathogens *in vitro*. Present evidence suggests that the active fraction of the *Rhodomela* extracts may be a brominated phenolic compound. Attempts are being made to purify the compound.

SEVERAL SPECIES of marine algae and two species of fresh-water algae have been shown to yield extracts that are mildly antibiotic *in vitro* against several species of pathogenic bacteria (1–3). The present report deals with the antibiotic activity of extracts prepared from *Rhodomela larix*, a red alga not previously studied in this manner.

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EXPERIMENTAL

Collection and Preservation.—*Rhodomela larix* is distributed along the Pacific coast of the United States from Alaska to central California and grows on rocks between the 2.0- and 0.5-ft. tide levels. As it occurs at Moss Beach, Calif., where the plants used in the present study were collected, thalli range from 2.5 to 10 cm. in length. The thalli were frozen within thirty minutes after removal from the ocean and were kept frozen until used in the laboratory, since extracts prepared from plants air dried at room temperature or dried at 80° are antibacterially inactive.

Preparation of Extract.—The plants were extracted, by the method previously described (1), for thirty hours, using peroxide-free, fat-extraction grade ether. Evaporation of the extract yielded noncrystalline, greenish-black, glittering flakes. Under favorable conditions, the yield of dry crude extract was about 0.3 Gm. per 100 Gm. of frozen thallus. Since *Rhodomela larix* contains about 85% of water, this represented a yield of 2% on a dry weight basis. The antibacterially active portion probably comprises only a small fraction of the dry crude product.

Microbiologic Testing.—In the routine for preliminary evaluation by the cylinder-plate technique, the extracts were tested in a 90:10 phosphate buffer-ethanol mixture having a *p*H of 6.8 and containing 10 mg. of extract per ml. The extract is soluble in this mixture only to the extent of 7 mg. per ml. at 25° but is more soluble at the incubator temperature (37.5°). Other tests in liquid media were made as required.

RESULTS

Microbiologic.—According to the experimental routine, four test organisms were used in evaluating new batches of extract, *viz.*, *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*), *Bacillus megatherium*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The average values obtained in cylinder-plate tests over a six-month period are shown in Table I.

TABLE I.—ACTIVITY OF ETHER EXTRACTS OF RHODOMELA LARIX (CYLINDER-PLATE TECHNIQUE)

Organism	Inhibition Zone, Diam., Mm.
M. PYOGENES var. AUREUS	17.3
B. MEGATHERIUM	14.0
E. COLI	11–16[a]
P. AERUGINOSA	11.2

[a] Depending on batch tested.

The response of *E. coli* was extremely variable and fluctuated in the course of routine monthly collections and assays. This may indicate the presence of more than one antibiotically active compound in the crude extracts, indicating that one compound or group of compounds affecting the other three test organisms is relatively constant in *Rhodomela* but that the concentration of a different component, which affects *E. coli*, is subject to seasonal and climatic conditions that influence the metabolic products of the seaweed. This hypothesis is being investigated.

The extracts are somewhat more active in cylinder plate tests at *p*H 6.0 than at *p*H 9.0, suggesting that the active compound is acidic.

Mycobacterium tuberculosis H37Rv was partially inhibited for twenty-one days in media containing 0.25 mg. of extract per ml. No surface-active agents were added to the test broth.

Agar plate tests (cork borer method with a well 12 mm. in diameter) showed that growth of *Trichophyton mentagrophytes* (wild strain) is partially inhibited. After incubation for seven days, the plates contained clear zones 32 mm. in diameter when the organism was challenged with the ethanol-phosphate mixture described above. Control plates were completely covered with the fungus after four days of incubation.

In other tests,[1] inhibiting concentrations of *Rhodomela* extracts in broth have ranged from 50 μg. per ml. for *M. pyogenes* var. *aureus*, *Mycobacterium phlei*, *M. tuberculosis* (607) and *M. avium* to 500 μg. per ml. for *Aerobacter aerogenes*, *Klebsiella pneumoniae*, *Salmonella gallinarum*, *Saccharomyces pastorianus*, *Candida albicans*, and *Trichophyton rubrum*.

Chemical.—The glassy flakes of crude extract melt, with decomposition, over the range 53–105°. They are insoluble in petroleum ether and in 5% HCl, and are only slightly soluble in benzene, in 5% $NaHCO_3$, and in distilled water, even upon heating. A pink flocculent precipitate forms when 5% HCl, containing 0.5 Gm. of suspended extract per 1.5 ml., is heated. The extract is soluble in ether, chloroform, 1,4-dioxane, ethanol, and methanol. It is also soluble in 5% NaOH but is completely inactivated by it. The extract is partially soluble (4.9 Gm./ml.) in phosphate buffer (*p*H 6.8) at 25°, and this solubility is improved by the addition of ethanol. There is no loss of activity in the buffer or buffer-ethanol mixture.

In view of the marine origin of the product (some seaweeds are known to accumulate iodine), it appeared important to determine whether the observed activity could be due to that element. The Microanalytical Laboratory of the University of California, Berkeley, reported the following results for duplicate analyses of *Rhodomela* extracts: iodine (volumetric), 0.05%; bromine (volumetric and gravimetric), 37.71%; chlorine (volumetric), none; total halogen (gravimetric), 37.63%; and total ash, approximately 1%. These figures suggest that the halogens present are primarily organic and that only bromine is present in significant amounts. The bromine value of approximately 38% indicates that the bromine content of the alga may be about 0.7% (dry weight basis). Control tests showed that the antibacterial activity of the extracts cannot be ascribed to iodine. Solutions of the extract immediately become bright bluish green when ferric chloride, T. S., is added, suggesting the possible presence of a phenolic compound. Since many plant products react with ferric chloride, this test cannot be considered definitive. Aqueous or dioxane solutions of the crude extract do not yield a solid bromide upon addition of bromine water, suggesting that if a phenolic compound is present, the 2-, 4-, and 6-positions are blocked.

Attempts to prepare phenol derivatives were uniformly unsuccessful, probably due to the high degree of impurity of the crude products. Among the derivatives attempted were aryloxyacetic acid (4), 3,5-dinitrobenzoate (5), phenylurethane (5), and bromide (5).

Stability.—The active material is not destroyed by boiling in ether (34.6°) or in methanol (64.7°) under reflux for two hours. The activity is lost completely, however, when the dry material is kept at 70° for one hour. At room temperature, the active fraction is stable for several days in alcoholic aqueous solutions (10:90 ethanol-phosphate buffer mixture), and when dry, is stable for several weeks.

SUMMARY

1. A crude ether extract that shows weakly inhibitory activity against several species of pathogenic microorganisms has been obtained from the red marine alga, *Rhodomela larix*

[1] Made in the Lilly Research Laboratories.

Among the organisms inhibited are *Micrococcus pyogenes* var. *aureus* (*S. aureus*), *Bacillus megatherium*, *Mycobacterium phlei*, *Myco. tuberculosis* (607 and H37Rv), and *Myco. avium*. Less sensitive are *Bacillus subtilis*, *Proteus vulgaris*, *Escherichia coli*, *Aerobacter aerogenes*, *Klebsiella pneumoniae*, *Salmonella gallinarum*, *Candida albicans*, *Saccharomyces pastorianus*, *Trichophyton mentagrophytes*, and *T. rubrum*.

2. Quantitative and qualitative tests of the crude extract suggest that it may contain a brominated phenolic compound, the activity of which is sensitive to both acid and alkali. The active principle is stable for at least two hours in methanol at 64°, but when dry the activity is completely destroyed in one hour at 70°.

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Effect of Potentiation or Inhibition of Acetylcholine on Intestinal Damage, Mortality, and Recovery after Acute Abdominal X-Radiation in the Rat*, †

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Autonomic drugs (atropine, neostigmine methyl bromide, tetraethylammonium chloride, Dibenamine, and nicotine) were injected subcutaneously in rats twenty minutes before and daily thereafter for periods of eighteen to thirty days following 600, 1,000, 1,250, and 1,500, r abdominal radiation. It has been shown that neither an increase nor a decrease in intestinal motility nor any other pharmacological action of the drugs employed furnish protection from the effects of abdominal radiation by decreasing mortality or prolonging the survival time, when compared with radiated saline-injected controls. Atropine increased the mortality rate at doses of 300 and 500 mg./Kg. A histological criterion of drug medication was unsatisfactory because of the rapid "turnover" of the intestinal epithelium.

REGAUD, Nogier, and Lacassagne (1) were the first to report the hypersensitivity of the epithelium of the gastrointestinal tract to the damaging effect of ionizing radiation, an observation later confirmed by the work of Warren and Whipple (2) and Quastler, *et al.* (3).

Larkin (4) prolonged the survival period of whole body-irradiated CF1 strain mice with intramuscular injections of 0.65 mg./Kg. of atropine given twenty-four hours before irradiation and daily for fifteen days following it. Burnett, *et al.* (5), found that 2 mg./Kg. of atropine injected intramuscularly into C57 strain mice failed to alter the survival pattern when given three to five minutes prior to 600 r of total body radiation, whereas intraperitoneal injections of acetyl-β-methylcholine bromide or chloride, or carbamylcholine chloride increased the twenty-eight-day LD_{50} radiation dose from 600 to 900 r. Conard (6) demonstrated that local X-ray irradiation of rat intestine stimulated cholinergic nerves. He also showed that drugs which tended to inhibit the action of acetylcholine also inhibit the X-ray response and, conversely, drugs that augment the action of acetylcholine also greatly augment the X-ray response. Bond, *et al.* (7), found the peak of deaths in abdominally irradiated rats to occur

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‡ The authors wish to thank Hoffman-La Roche, Inc., for supplying neostigmine methyl bromide, Parke, Davis and Co. for tetraethylammonium chloride, and Smith, Kline and French Laboratories for Dibenamine; and Dr. A. E. Lewis and Mrs. D. N. Highby for the histological preparation and examination of the various tissues.

from four to five days after exposure, and that of irradiated abdominally shielded animals to occur between the ninth and twelfth postradiation day. Quastler, *et al.* (3), reported the death peak in mice given 400 r of whole body radiation to be at about eleven and one-half days; when the radiation dose was increased, the peak of deaths fell to three and one-half days. These two lethal periods were designated as characteristic values of the response spectrum of the mouse. From the results of partial or regional irradiation experiments, they concluded that irradiation of any large portion of the intestine was necessary and sufficient to produce the three and one-half day mortality process. The present investigation was undertaken to determine what protective effect, if any, the various autonomic agents (atropine, neostigmine methyl bromide, nicotine, tetraethylammonium chloride, and Dibenamine) might have on rats subjected to varying doses of X-ray irradiation administered to the abdomen.

EXPERIMENTAL

Tables I, II, and III and Fig. 1 show the various groups and the drugs administered to 446 CFW strain rats of either sex having an initial body weight of approximately 175 Gm. Four experiments were performed in which the drug and radiation dosages varied. All drugs were injected subcutaneously twenty minutes prior to irradiation and daily thereafter for periods of eighteen to twenty-eight days. The animals were anesthetized during irradiation by intraperitoneal injections of 30 or 45 mg./Kg. of sodium pentobarbital. The non-radiated controls were similarly treated. Local abdominal radiation was administered to the rats in groups of twelve while they were securely taped upon two perforated lead shields 8.5 x 24 x 0.25 in. Radiation was applied to the abdomen through holes in the shields (2.5 x 3.2 cm., tapered, = 7.6 sq. cm.) and to an area below the rib cage, tapering toward, but excluding, the spinal column.

The radiation was administered with a 250-KVP Picker Industrial Unit whose output was calibrated before and after each experiment with a Victoreen Thimble radiation meter. Radiation characteristics were as follows: 250 kv.; 15 ma.; TSD varied from 50 to 100 cm.; filters, 0.21 mm. Cu inherent, 0.5 mm. Cu parabolic, and 1.0 mm. Al; HVL 1.83 mm. Cu; r/minute measured in air varied from 38 to 45.5.

Three intestinal sections (the second-inch segment below the pylorus and a similar segment above and below the cecum) were taken for histological study from the animals that died during the experiment and from animals sacrificed at its conclusion. When the respective segments were tied off after being filled with barium, roentgenographs showed that they were within the exposure area of the shield.

Body weight curves were obtained as an over-all indication of the response of the animals to the experimental procedures.

RESULTS

Experiment I.—The medication produced a variety of untoward reactions. Atropine injections were corrosive. Dibenamine injections followed by irradiation produced extensive edema over the forequarters; subsequent injection sites were never free of edema and, like the atropine injection, resulted in a leather-like consistency of the skin. Nicotine administration caused tetanic convulsions followed by depression, and these reactions neither increased or decreased in intensity throughout the experimental period. Neostigmine methyl bromide produced large, soft, wet, but formed, stools in contrast to the small, hard, dry stools of the atropinized animals. Mortality figures are shown in Table I. No deaths occurred during the experiment in the saline, neostigmine methyl bromide, or nicotine-irradiated (R) and non-irradiated (NR) groups. The mortality was 100% in the atropine R and NR groups, 12.5% in the tetraethylammonium R group, and 50% in the NR animals, whereas a 12.5 mortality occurred in the Dibenamine NR group. Deaths occurred throughout the experimental period and have been attributed to drug toxicity rather than to the effect of radiation.

Histological study of intestinal sections of radiated medicated and radiated non-medicated animals of all drug groups that died two to six days after irradiation showed an intact intestinal mucosa, some distortion of the crypt epithelial alignment, debris at the base of the crypts, and minor irregularities in the size and staining characteristics of the epithelial nuclei. Intestinal sections of animals that died seven to twenty-eight days after radiation were essentially normal. There were no histological changes that could be attributed to the medication alone.

Experiment II.—Forty-eight rats were medicated as in the first experiment, given 600 r total body radiation, and sacrificed three, four, five, and six days after irradiation. Characteristic intestinal tissue changes due to radiation were noted in all drug groups on the third and fourth days but were diminished in intensity or absent on the fifth and sixth postradiation days. No histological changes that could be attributed to the drugs were noted.

Experiment III.—Results were the same as for Experiment I except that the doses of medication were reduced, and discontinued after eighteen days (see Table II). Two local abdominal radiation dosages were used, 1,000 and 1,250 r.

Toxicity and untoward reactions following medication were present but less intense than those seen in the first experiment. Atropine remained the most toxic drug, with the condition of the animals the poorest and the mortality the highest. The increased abdominal radiation dosage lowered the body weight curves and depressed the rate of body weight recovery of all irradiated animals. The intestinal content of the rats that died was pink-tinged, but localized hemorrhagic or necrotic areas were not observed in the mucosa. Furthermore, histological sections of the intestines from animals sacrificed at the end of the experiment were essentially normal. No histological changes were observed that could be attributed to the medication.

TABLE I.—EFFECT OF MEDICATION ON MORTALITY AND BODY WEIGHT AFTER 600 r ABDOMINAL IRRADIATION IN RATS

Subcutaneous Medication[a] and Dose	Group	Total Mortality,[b] 28 Days	Effect on Body Wt., as Av. % Initial Wt. — Days 6	12	18	28
Saline, 0.9%, 0.2 cc.	Irradiated	0/9	+10	+29	+48	+67
	Non-irradiated	0/7	+14	+27	+30	+55
Atropine sulfate, 500 mg./Kg.	Irradiated	13/13	−18	−25	−40	−30
	Non-irradiated	16/16	−12	−12	−11	...
Neostigmine methyl bromide, 0.1 mg./Kg.	Irradiated	0/7	+13	+27	+42	+65
	Non-irradiated	0/5	+17	+36	+50	+68
Tetraethylammonium chloride, 50 mg./Kg.	Irradiated	1/8	+15	+28	+48	+63
	Non-irradiated	4/8	+13	+25	+43	+59
Nicotine, 10 mg./Kg.	Irradiated	0/8	+8	+19	+26	+44
	Non-irradiated	0/5	+6	+17	+22	+37
Dibenamine, 100 mg./Kg.	Irradiated	0/6	+4	+13	+18	+40
	Non-irradiated	1/8	+7	+13	+20	+39

[a] Medicated for 28 days.
[b] Total mortality = no. dead/total no. in group.

TABLE II.—EFFECT OF MEDICATION ON MORTALITY AND BODY WEIGHT AFTER 1,000 AND 1,250 r ABDOMINAL IRRADIATION IN RATS

Subcutaneous Medication[a] and Dose	Group	1,000 r Dose: Total[c] Mortality, 28 Days	Av. % Effect on Body Wt. — 6 Days	12 Days	18 Days	28 Days	1,250 r Dose: Total Mortality, 28 Days	Av. % Effect on Body Wt. — 6 Days	12 Days	18 Days	28 Days
Saline, 0.9%, 0.2 cc.	R[b]	0/7	−2	+17	+32	+50	0/10	−18	−11	−2	+12
	NR[b]	0/7	+12	+33	+45	+61	0/9	+7	+12	+18	+24
Atropine sulfate, 300 mg./Kg.	R	8/8	−36	−7	+3	...	9/9	...	...	...	...
	NR	7/8	−7	−1	+1	...	2/5	−3	−5	−6	−2
Neostigmine methyl bromide, 0.06 mg./Kg.	R	0/8	+1	+19	+30	+49	1/9	−17	−6	+6	+18
	NR	0/7	+12	+32	+47	+65	0/10	+2	+13	+20	+25
Tetraethylammonium chloride, 30 mg./Kg.	R	3/9	−16	+2	+17	+45	1/10	−20	−13	−8	+9
	NR	0/7	+13	+37	+51	+63	0/10	+4	+12	+17	+22
Nicotine, 6 mg./Kg.	R	0/8	−14	−2	+14	+35	2/10	−17	−15	−8	+1
	NR	0/8	+9	+19	+29	+45	1/10	+5	+6	+9	+16
Dibenamine, 60 mg./Kg.	R	0/7	−3	+10	+20	+45	0/8	−12	−9	−1	+12
	NR	0/7	+7	+14	+27	+49	1/9	+2	+6	+8	+13

[a] Medicated for 18 days.
[b] R—irradiated; NR—non-irradiated.
[c] Total mortality = no. dead/total no. in group.

TABLE III.—EFFECT OF MEDICATION ON MORTALITY AND BODY WEIGHT AFTER 1,500 r ABDOMINAL IRRADIATION IN RATS

Subcutaneous Medication[a] and Dose	Group	Mean Survival Time,[b] Days	Total Mortality,[c] 30 Days	Effect on Body Wt., as Av. % Initial Wt. — 6 Days	12 Days	18 Days	30 Days
Saline, 0.9%	1 Med. rad.	15.4	6/10	−22	−28	−19	−3
	2 Med. non-rad.	...	0/10	+5	+8	+12	+18
Atropine sulfate, 100 mg./Kg.	3 Med. rad.	5.7	9/9	−30	−33	...	...
	4 Med. non-rad.	...	0/10	−3	−1	+3	+14
Atropine, 25 mg./Kg.	5 Med. rad.	9.5	10/10	−25	−42	...	...
	6 Med. non-rad.	...	0/10	+2	+4	+6	+17
Neostigmine methyl bromide, 0.06 mg./Kg.	7 Med. rad.	11.8	7/9	−19	−30	−14	+12
	8 Med. non-rad.	...	0/10	+7	+11	+14	+21

[a] Medicated for 18 days.
[b] Calculated by Goodwin's formula (10): $MST = \frac{1}{L_0}(\frac{1}{2}L_0 + L_1 + L_2 \ldots L_{n-1} + \frac{1}{2}L_n)$ where L is the days of treatment and n is the number of animals in a group.
[c] Total mortality = no. dead/total no. in group.

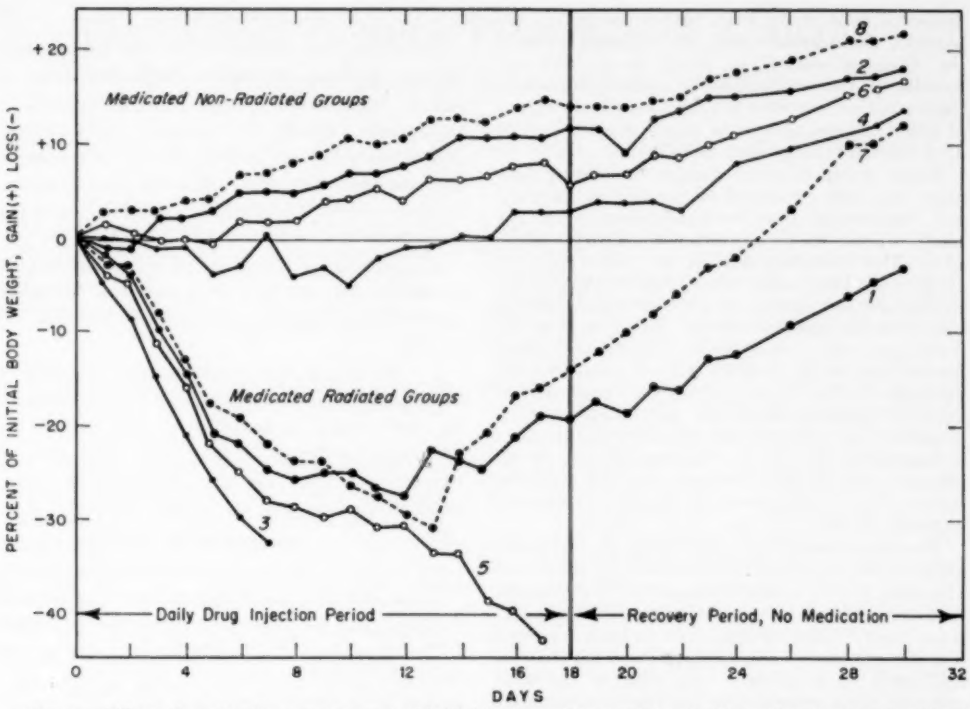

Fig. 1.—Effect of medication on per cent of initial body weight in abdominally radiated and nonradiated rats. 1, radiated, received 0.2 cc. of saline; 2, nonradiated, received 0.2 cc. of saline; 3, radiated, received 100 mg./Kg. of atropine sulfate; 4, nonradiated, received 100 mg./Kg. of atropine sulfate; 5, radiated, received 25 mg./Kg. of atropine sulfate; 6, nonradiated, received 25 mg./Kg. of atropine sulfate; 7, radiated, received 0.06 mg./Kg. of neostigmine methyl bromide; 8, nonradiated, received 0.06 mg./Kg. of neostigmine methyl bromide.

Experiment IV.—This experiment was designed to evaluate the effect of inhibition and potentiation of acetylcholine on the response of animals to 1,500 r abdominal radiation. The results are shown in Table III and Fig. 1.

The comparatively nontoxic drug doses used produced neither deaths nor significant variation in the body weight curves of medicated nonradiated animal groups 2, 4, 6, and 8 (Table III and Fig. 1), and the untoward drug reactions in atropine group 6 were negligible. The medicated radiated groups sustained a total mortality of sufficient magnitude to demonstrate the ineffectiveness of repeated daily medication to increase the number of survivors following irradiation above that of the saline controls. The mortality of saline control group 1 was 60%, that of groups 3 and 5 was 100%, and that of group 7 was 77%. Medication decreased the survival time of irradiated rats below that of the saline controls, illustrated by the mean survival time of 15.4 days for group 1, 5.7 days for group 3, 9.5 days for group 5, and 11.8 days for group 7. Death frequencies were peaked on the seventh post-radiation day in group 3, at which time five animals died. Deaths in the other groups were distributed from four to eighteen days after X-ray. Every animal sacrificed at the end of the recovery period showed an essentially normal intestinal mucosa grossly and histologically.

DISCUSSION

Neither an increase or a decrease in intestinal motility nor any other pharmacological action of the autonomic drugs employed furnished protection from the effects of abdominal radiation by decreasing total mortality or prolonging the survival time, when compared with radiated saline-injected controls. Atropine medication either increased animal susceptibility to the abdominal radiation or the radiation increased the sensitivity of the animals to the drug, thus increasing the mortality rate and total mortality. This observation is in variance with the protective action of atropine to the effects of ionizing radiation reported by Larkin (4) but is in agreement with the findings of Burnett, *et al.* (5). However, species differences between mice and rats may be involved and the present experiments would not detect or resolve such differences in response.

Bond, *et al.* (7), reported a thirty-day LD_{50} X-ray dosage of 1,025 r for rats with the total abdomen exposed. In the present experiments, 600, 1,000, and 1,250 r of local abdominal irradiation produced no mortality in the control groups, whereas 1,500 r gave a mortality of 60%. The differences in radiation responses are probably due to the fact that the method of shielding used in the present study

protected most of the liver, half of the spleen, the kidneys, the adrenals, and the skeleton, whereas the shielding method of Bond, *et al.*, did not. Results of this study confirm their observations that radiation mortality is a function of the amount of tissue and the number of organs exposed as well as of the total energy absorbed (7).

Tissue susceptibility to damage by ionizing radiation has been expressed in the law of Bergonie and Tribondeau that "radiosensitiveness of any tissue depends on its reproductive activity" [Boyd (8)]. The reproductivity of rat intestinal epithelium has been reported by Leblond and Stevens (9) to have an average duration of mitosis of 1.13 hours, and an average cell life of 1.57 days in the duodenum and 1.35 days in the ileum. These figures refer to the time for the cell to move from the crypt to the villi tip. Thus the "turnover" of the rat intestinal epithelium *per se* enhances its sensitivity to radiation but precludes employment of histological changes as a possible criterion of the efficacy of medication following limited radiation damage when studied for an eighteen to twenty-eight-day period.

The higher the abdominal radiation was increased, the lower the body weight curves fell, and the slower the onset and the more depressed the body weight recovery. The body weight recovery of neostigmine methyl bromide-medicated animals, radiated and non-radiated (Tables I, II, III and Fig. 1), was equivalent to or greater than that of the non-radiated saline controls and has been attributed to the beneficial effect of increased intestinal motility. When the body weight curves of the saline control groups were computed in the same manner as those of Bond, *et al.* (7), they were similar but less depressed because of the smaller area of abdominal radiation.

SUMMARY

1. Under the conditions of this experiment, autonomic drug medication employing atropine sulfate, neostigmine methyl bromide, tetraethylammonium chloride, Dibenamine hydrochloride, and nicotine did not prolong the survival time or decrease mortality above that observed in saline-treated animals following X-ray irradiation of the abdomen.

2. Atropine medication not only failed to give protection but actually increased the mortality rate and total mortality at doses of 300 and 500 mg./Kg.

3. No histological changes were observed in the intestinal sections that could be attributed to the respective drug medication. Furthermore, a histological criterion of drug medication efficacy was unsatisfactory because of the rapid "turnover" of the intestinal epithelium.

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The Isotope Dilution Procedure of Analysis. I. Historical and Literature Survey*

By JOHN J. PINAJIAN, JOHN E. CHRISTIAN, and WALTER E. WRIGHT

The isotope dilution procedure of analysis, a relatively new technique, offers intriguing possibilities and is of sufficient importance to warrant special attention at this time. The history of the procedure and a survey of the literature are presented.

AT THE present time there are many accurate techniques and highly specialized instruments, developed over a number of years, which make possible the quantitative analysis of individual and multiple component mixtures. Most of them are quite adequate for their intended purposes; however, there are still a number of components and mixtures of components which cannot be satisfactorily analyzed. Research workers must be constantly alert for the development of any new method which may be of value in solving new and unusual analytical problems.

A recently developed method of analysis which will ultimately find particular application among other well known procedures is "isotope dilution." This method is a useful and powerful new tool which may often be applied to analytical problems that cannot be solved adequately by any other means.

HISTORICAL DEVELOPMENT

The introduction of the "isotope dilution" technique as an analytical tool was actually an outgrowth of the struggle to establish the validity of Prout's hypothesis. At the beginning of the 19th century, Dalton had established what was then the modern atomic theory: "Atoms of the same element are similar to one another and equal in weight." In 1815 Prout, the physician who had discovered the presence of HCl in the stomach, published anonymously a hypothesis that hydrogen was the basis of structure and that the whole-number rule applied to the atomic weights of the elements: "The atomic weights of elements are equal to whole numbers times the atomic weight of hydrogen."

Meanwhile, chemists extended the accuracy of atomic weights beyond two figures. The chemical atomic weights were based on natural oxygen (which we now know to be a mixture of $O^{16,17,18}$) with an atomic weight of 16.00000, and were average weights of something in the order of 10^{22} atoms. Naturally certain discrepancies became obvious. In the face of these difficulties, Dalton's theory was questionable but Prout's hypothesis was discarded. Dalton's theory was not questioned again until the end of the 19th century. As atomic weights became known with increasing accuracy, it was apparent that both theories could not be right; either elements had fractional masses or different masses with whole numbers.

In 1907, R. B. Boltwood, a physical chemist working at Yale University, discovered ionium the parent of radium. He found that the properties of this new element coincided exactly with those of thorium. Soddy, another chemist working in this new field, wrote to Rutherford who was then at Manchester, "The thorium and ionium remain together when once mixed, and no chemical process is capable of separating them again. The two are chemically identical" (1). Such elements are now called isotopes. Boltwood continued in his futile attempt to separate these two elements, not grasping the significance of the situation.

The method of isotope indicators and isotope dilution had its origin at the University of Manchester, which was under the direction of Rutherford.

At this time, the chief source of radium was the town of Joachimstal in Czechoslovakia. The Austrian Government, owner of the mines, supplied Rutherford with radium and its by-products, and presented him with several hundred kilograms of radium D, the useful radioactive by-product. Unfortunately, it was associated with very large amounts of lead which absorbed the radiations and made the material useless. Hevesy (2) recalls meeting Rutherford in the basement laboratory where the material was stored. "He addressed me in his friendly informal way, saying: 'My boy, if you are worth your salt, you try to separate radium D from all that lead.' In those days, I was an enthusiastic young man and, immediately starting to attack the problem suggested to me, I felt quite convinced that I would succeed. However, although I made numerous attempts to separate radium D from lead and worked for almost two years at this task, I failed completely. In order to make the best of this depressing situation, I decided to use radium D as an indicator of lead, thus profiting from the inseparability of radium D from lead." This was the first instance of the utilization of isotopes as tracer substances.

To obtain a larger supply of radium than he had at his disposal at Manchester, Hevesy went to the Vienna Institute of Radium Research in 1912 and worked in collaboration with Paneth, who was then an assistant at the Institute. The first application of labeled lead as an analytical tool (3) was the determination of the solubility of some very slightly soluble lead compounds such as lead chromate. The well-known Nernst formula was proved; hydrides were investigated (4, 5), Maxwell's self-diffusion in liquid lead (6), solid lead (7, 8) and salts (9) was studied, and Arrhenius' theory of electrolytic dissociation was proved correct (10).

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The early use by Hevesy and his co-workers of naturally occurring radioactive lead (radium D) in the study of chemical principles later led to the use of the isotope dilution procedure as a quantitative analytical tool. Although the basis was laid, isotope utilization did not hold a prominent place in experimental chemistry until the 1930's, due to the difficulty of separating natural isotopes from their contaminants in nature, and the fact that artificially produced isotopes (11, 12) were not available. However, with the isolation of deuterium by Urey, *et al.* (13) in 1932, and the production of artificially produced isotopes after the construction of the first cyclotron in 1932 (14), investigation of the use of isotopes rapidly increased. Hevesy and Hobbie (15), in 1932, were the first to report the use of a radioactive isotope to solve an analytical problem, and Hevesy and Hofer (16) introduced and pioneered the isotope dilution technique in 1934 by determining and tracing crudely the amount and fate of water in the human body, using deuterium-enriched water (heavy water). The work of Hevesy and Paneth in the field of isotopes began about 1910, and as a result of their experience in this field, they published "A Manual of Radioactivity" (17) and a complete review of progress with tracers (18) in 1937.

Although isotope dilution analysis was introduced in 1934, it was not accepted until 1940 when the work of Rittenberg and Foster (19) on the analysis of proteins for amino acids, using N^{15}, and the investigations of Ussing (20) revived interest in this procedure. From this time, the possibilities of the method were apparent to workers in almost every field of chemistry. As more isotopes became available in this decade, the method came into greater use, but is still limited because of lack of know-how and the necessity of special equipment.

LITERATURE

The work of Rittenberg and Foster (19), which stimulated the use of isotope dilution in the early 1940's, was based on the utilization of deuterium labeled palmitic acid for the quantitative evaluation of the palmitic acid content of animal fat. About this time Ussing (20) determined the leucine content of hemoglobin by a similar method, using deuterioleucine.

Schoenheimer, Ratner, and Rittenberg (21) studied the metabolism of body proteins of the rat with N^{15} and deuterium labeled *l*(−)-leucine. The fate of N^{15} labeled tyrosine was also determined by this technique (22).

In 1940 Graff, Rittenberg, and Foster (23) reported the utilization of N^{15} labeled *dl*-glutamic acid in the study of the glutamic acid content of malignant tumors. By the use of isotope dilution, it was definitely proved that malignant tissue contains *l*(+)-glutamic acid in significant amounts, a point which had previously been in dispute.

Flagg and Wiig (24) cited several advantages and disadvantages of the isotope dilution procedure of analysis. Chargaff, Ziff, and Rittenberg (25, 26) in 1941 and 1942 reported using N^{15} to study the composition of tissue phospholipids, and Shemin (27) employed it to determine several amino acids in bovine and human serum albumins. Foster (28), also using N^{15} and isotope dilution procedures, analyzed hemoglobin and β-lactoglobulin for amino acid content.

Süe (29, 30) was the first to report the use of a radioactive isotope in isotope dilution procedures. He estimated the amount of potassium in a sample which also contained lithium and sodium by adding radioactive potassium of known specific activity, precipitating the potassium as perchlorate, and separating it; from the new specific activity, the amount of inactive potassium initially present was calculated.

Shemin and Foster (31) discussed the isotope dilution method of amino acid analysis with N^{15} and estimated the errors involved in the method to be about 1–2%. The separation of the *d* and *l* enantimorphs of a sample and the calculations for the degree of racemization were described. Analytical procedures were given for tyrosine, leucine, phenylalanine, arginine, lysine, glutamic acid, aspartic acid, and glycine. A summary of the results with analytical data for the seven *l*-amino acids in cattle fibrin, horse hemoglobin, β-lactoglobulin, bovine serum albumin, and human albumin was presented.

In 1946 Henriques and Margnetti (32) reported the analysis of three individual components of a mixture of dibenzyl sulfide, sulfoxide, and sulfone in which S^{35} was employed in the usual isotope dilution procedure. At the same time, the possibility of the use of inverse isotope dilution procedures was suggested and formulas were given.

Shortly thereafter, Keston, Udenfriend, and Cannan (33) published preliminary data on the use of the inverse isotope dilution procedure by which a simple mixture of amino acids was analyzed by first forming a derivative with I^{131} labeled pipsyl chloride (*p*-iodophenylsulfonyl chloride) of known specific activity. Unlabeled derivative was then added and purified to constant specific activity after mixing. The amount of amino acid originally present was then easily calculated.

Moore (34) discussed the determination of total body water by deuterium oxide and of body solids using radioisotopes of sodium and potassium.

Gest, Kamen, and Reiner (35) derived an exact set of equations to be used in isotope dilution analyses in 1947, and discussed the validity of the approximate equations already in use.

Keston, Udenfriend, and Levy (36) reported using the inverse isotope dilution analysis in combination with paper chromatography to separate the I^{131} labeled pipsyl derivative, adding S^{35} labeled derivative as carrier, and purifying the compound to a constant I^{131}/S^{35} ratio.

Radin (37) outlined and reviewed several aspects of analysis by isotope dilution and inverse isotope dilution, including calculations, precision considerations, purity, advantages, disadvantages, and applications.

In 1948 Block and Anker (38) reported a method of inverse isotope dilution which depended on the determination of two aliquots of an unknown by using different quantities of labeled compound. The authors claimed that this procedure gave more precise results.

Hindin and Grosse (39) in 1948 published a modification of the isotope dilution procedure which was suitable for the determination of trace elements. Data on the analysis of pure compounds were given

with comparative figures for both Kjeldahl and isotope dilution procedures.

Winnick, *et al.* (40, 41), working with C^{14}, followed the distribution and utilization of labeled glycine in the process of amino acid incorporation by the protein liver homogenate.

Grosse, Hindin, and Kirshenbaum (42–44) made determinations of oxygen, carbon, and nitrogen in organic compounds by means of isotope dilution, utilizing the stable isotopes O^{18}, C^{13}, and N^{15}. The method does not require quantitative determination or specificity of reaction.

Isotope dilution was employed in theoretical chemistry by Wheland and Schwarz (45) in an attempt to find crossed products in the benzidine rearrangement. By adding a possible crossed product as a carrier, sufficient material was isolated in preliminary work to show that the particular cross product in mind did not exist. The technique offers a wide range of application in this relatively untouched field.

Keston, Udenfrend, and Cannan (46) gave a complete description of their inverse isotope procedure for amino acids, using pipsyl chloride. They discussed the precision and the validity of the method.

Yankwich (47) reviewed the latest developments in isotopic dilution methods and discussed the criteria of purity in some detail.

Gross and Leblond (48, 49) employed isotope dilution and filter paper chromatography to demonstrate the presence of free thyroxine in unhydrolyzed thyroid tissue. Although they were able to demonstrate that the free thyroxine concentrate of the gland was much greater than the plasma, they were unable to show the actual quantity of free thyroxine present.

Siekevitz and Greenberg (50), using a combination of inverse isotope dilution and carrier technique, investigated the biological formation of serine from glycine.

Raben (51) described a microdetermination of iodine with radioactive iodine by a modified isotope dilution technique. Labeled iodine was produced by reacting the unknown in the form of iodate with an excess of radioactive iodide of known specific activity. Iodine was extracted with carbon tetrachloride and the radioactivity in carbon tetrachloride was determined by gamma ray counting.

Keston, Udenfriend, and Levy (52) reported on the analysis of amino acids by I^{131} labeled pipsyl chloride, separating the derivatives by paper chromatography, and using S^{35} labeled pipsyl amino acids as carriers.

Berenbom, Sober, and White (53), utilizing the methods of Rittenberg and Foster (19) and Block and Anker (38), derived a procedure for N^{15} urea in rat urine in which isotopically labeled carrier is used for the simultaneous quantitative and isotopic analysis by isotope dilution. The method is applicable where both the quantity and isotopic concentration of a compound are unknown, as in the case of intermediates.

Wright (54) analyzed iodoprotein (protamone) for thyroxine by the isotope dilution technique.

Ropp (55) demonstrated a particularly valuable application to determinations of successive yields in a series of organic reactions where the scale is so small that isolation and identification of the successive products is impossible. The studies entail the synthesis of malonic acid-2-C^{14} and diethyl malonate-2-C^{14} by independent methods.

Ennor and Rosenberg (56) discussed common errors involved in determining specific activity and turnover rates of radioactive isotopes as applicable to P^{32} counting.

A unique application in which 11 monoamino acids in a single milligram of protein were subjected to analysis was reported by Velick and Udenfriend (57). Two labeled compounds (I^{131}, S^{35}) and chromatographic procedures with pipsyl chloride derivatives yielded a sensitive method of analysis for many amino acids in extremely small samples of proteins and enzymes (58). The method has been extended to analysis for protein amino end groups with applications to bovine insulin, horse hemoglobin, and rabbit muscle aldolase (59).

Labeled penicillin has been prepared and applied to the determination of penicillin G in broth as well as in commercial crystalline samples by Craig, Tindall, and Murray (60).

Dziewiatkowski (61, 62) has attempted to study the S^{35} deposition in articular cartilage by isolating chondroitin sulfate from cartilage by the addition of nonlabeled chondroitin sulfate as a carrier and estimating S^{35} deposition by isotopic dilution principles.

Ropp (63) reviewed the uses of isotopic carbon in organic research in two general classes: essential uses and non-essential uses. Mention was made of the dilution method of analysis and examples were given of the few organic problems which had incorporated the use of isotopic carbon.

The metabolism of glucose (64, 65), histamine (66), hypoxanthine (67, 68), barbiturates (69, 70), uric acid (71), and 8-azaguanine (72) and the mechanism of nitrogen storage (73, 74) have been studied using isotope dilution principles.

Maynor and Collins (75) described the use of the double dilution method for the simultaneous determination of yield and activity of radioactive compounds in their work on the synthesis of chrysene-5,6-C^{14}. The yield of a product which resisted all attempts at purification was calculated.

Hutchins and Christian (76) reported the separation of sulfonamide mixtures by ion exchange chromatography and used the method in combination with isotope dilution for accurate analysis.

The availability of high specific activities, reported, for example, by Grosse, *et al.* (77), and Rosenblum, *et al.* (78), will increase the range and sensitivity of the isotope dilution analysis.

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The Isotope Dilution Procedure of Analysis. II. Procedure*,†

By JOHN E. CHRISTIAN and JOHN J. PINAJIAN

Three procedures of analysis, based on the isotope dilution method, are described and the limitations of each are discussed. The equations used for calculations are given.

THE "isotope dilution" method of analysis basically consists of three general types of procedures: (*a*) the "direct isotope dilution" procedure; (*b*) the "inverse isotope dilution" procedure; and (*c*) the "double isotope dilution" procedure. Although the processes are based on the same fundamental principles, they differ sufficiently in procedure, advantages, disadvantages, accuracy, etc., to be considered separately. As the names imply, the methods depend on the measured dilution of isotopically labeled atoms or molecules of a compound. As in all isotopic tracer work, with several elements there is a choice of the isotope which may be used.

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† A previous paper (3) in this series discusses the history of isotope dilution procedures and presents a survey of the literature.

EXPERIMENTAL

Choice of Isotope for Isotopic Dilution Analysis.—When a choice exists between the use of a rare stable isotope or a radioactive isotope, the following factors must be carefully evaluated: (a) Availability of measuring device; (b) availability of isotope; (c) the extent of dilution expected; (d) time required for experiment; (e) cost of isotope; and (f) problems of labeled compound synthesis.

The use of stable isotopes requires a mass spectrometer, whereas with radioactive isotopes, a Geiger-Müller counter assembly, which costs less and saves time, is employed. Radioactive isotope labeled compounds can be detected at much greater dilutions than the corresponding stable isotope labeled compounds, yet the latter represent permanent reagents which is not true of radioactive isotopes with short half-life characteristics.

The only stable isotopes readily available and measurable are H^2, C^{13}, N^{15}, O^{18}, and S^{34}. There are no usable radioactive isotopes of nitrogen and oxygen.

The accuracy of the analysis is not appreciably affected by the choice of isotope. However, when radioactive isotopes are used, the accuracy varies with the time of counting but is no more accurate than stable isotope measurement even under the best counting conditions. The computations necessary with stable isotope analysis are more involved and time consuming.

Direct Isotope Dilution Analysis.—This method of analysis consists essentially of adding a known weight of isotopically labeled ions or molecules of a compound (the isotopic content must be accurately known) to a mixture containing an unknown quantity of the unlabeled ions or molecules of the same compound, mixing completely, isolating a portion of the purified material, and accurately determining the isotopic content. Since the unlabeled material analyzed is chemically identical with the labeled substance added, thorough inseparable mixing occurs; and the isotope concentration of the labeled substance is reduced in direct ratio to the amount of unlabeled material originally present. From the isotopic concentration values before and after mixing, the amount of unlabeled material can be calculated. The distinct advantage of such a procedure is that *quantitative isolation is not necessary* at any stage of the analysis; it is only necessary to isolate a pure sample in a measurable quantity which can then be subjected to isotopic analysis.

The isotopic analysis in the case of radioactive isotopes involves the determination of the specific activity, an expression of activity per unit weight. If an amount of substance weighing G_1 mg. has an activity of A_1 counts per minute, the initial specific activity (Sp. Ac.$_i$) = A_1/G_1. When this material is added to or mixed with G_2 mg. of inactive material, the final specific activity (Sp. Ac.$_f$) = $A_1/(G_1 + G_2)$.

Solving the two equations simultaneously for the desired unknown weight, G_2, we arrive at:

$$G_2 = G_1\left(\frac{\text{Sp. Ac.}_i}{\text{Sp. Ac.}_f} - 1\right)$$

where G_1 = weight of labeled material, G_2 = weight of original unlabeled material, Sp. Ac.$_i$ = specific activity of initially labeled material (G_1), and Sp. Ac.$_f$ = specific activity of final mixture of ($G_1 + G_2$).

The use of high radioactive isotope concentrations would introduce a slight error in this calculation; however, high concentrations are not used since the sensitivity of detection of radioactive isotopes is great.

The isotopic analysis in the case of stable isotopes involves the determination of the isotopic ratio by the mass spectrometer. This is expressed as the atom % of the rare isotope or, more commonly and conveniently, as the atom % excess which is obtained by subtracting the normal isotopic abundance from the observed abundance.

Since detection is not as sensitive with stable as with radioactive isotopes, high stable isotopic concentrations are often used and affect the molecular weight of the substance appreciably. H^2 and N^{15} are not infrequently used in concentrations as high as 99 and 32 atom %, respectively, whereas radioactive isotopes are used in atomic concentrations well below 1%. The change in molecular weight of the labeled and unlabeled material in the case of stable isotopes should be corrected. Otherwise the formula for calculation using stable isotopes is essentially the same as for radioactive isotopes:

$$G_2 = G_1\left(\frac{E_o}{E} - 1\right)\frac{M}{M^1}$$

where G_2 = weight of unlabeled material, G_1 = weight of labeled material, E_o = atom % excess of labeled material, E = atom % excess of isolated material, M = molecular weight of unlabeled material, and M^1 = molecular weight of labeled material.

If low isotopic concentrations are used, the factor M/M^1 may be disregarded.

It should be emphasized that the direct isotope dilution procedure is not capable of detecting submilligram quantities of materials. In order to determine the specific activity of the isolated material with any degree of accuracy, in most cases 5–100 mg. of material must be isolated in a pure state. It is advisable to run more than one sample to improve precision; thus, in many instances, more pure isolated material is required. Often much material is lost in the steps necessary to assure adequate purity. Also, the amount of labeled material which can be added is limited by the amount of unlabeled material which is being determined, since the per cent error in the analysis depends partially upon the ratio between the weight of labeled compound added and unlabeled compound present. Radin (1) has published a table relating this ratio (G_1/G_2) to the per cent error.

Error in G_2, %	G_1/G_2
Infinity	Infinity
10	9
3	2
1.5	0.5
1.1	0.1
1.01	0.01

This means that if the per cent error is to be kept within 3%, the amount of labeled compound cannot exceed twice the amount of unlabeled material present. In cases of analysis of small amounts of unlabeled material, the yield of isolated material may be too small to purify adequately.

Assuming that a favorable G_1/G_2 ratio is used, the greatest uncertainty in the direct isotope dilution procedure is the question of the purity of the substance isolated from the mixture. There is usually no difficulty on this score with the labeled compound to be added, since adequate steps can be taken for purification. When using stable isotopes as the label, the sample for isotope ratio analysis must be free from any contamination which contains the element in question. No error is introduced by the presence of material which does not contain the element. If radioactive isotopes are used, any type of impurity will result in error. The steps for establishing purity and identity are discussed by Yankwich (2).

Table I lists the criteria considered necessary for direct isotope dilution analysis, and Table II lists some of the advantages of the method.

TABLE I.—CRITERIA NECESSARY FOR DIRECT ISOTOPE DILUTION ANALYSIS

1. Instruments of measurement must be available.
2. Exchange reactions of labeling atoms of a compound must be absent under the conditions of the analysis.
3. Isotopic labeled compound must be available or prepared with sufficient isotopic label to withstand dilution.
4. Milligram or greater quantities of the material being analyzed are necessary for successful analysis.
5. It must be possible to isolate a measurable quantity of the pure compound to determine its specific activity or isotopic ratio.
6. An error in the order of 1% is to be expected under usual conditions of the analysis.

TABLE II.—ADVANTAGES OF THE DIRECT ISOTOPE DILUTION ANALYSIS

1. The method can be used for many analytical problems which cannot be solved by other methods.
2. Quantitative isolation is not necessary.
3. It is often more rapid and more convenient, particularly if radioactive isotopes are used.
4. No doubt exists about the identity of the element being measured.
5. Accuracy can be varied with radioactive labels, depending on the time of counting and the G_1/G_2 ratio.

Inverse Isotope Dilution Analysis.—Inverse isotope dilution, as the name implies, is the reverse of direct isotope dilution analysis. The method consists essentially of adding a known weight of unlabeled ions or molecules of a compound to a mixture containing an unknown quantity of the labeled ions or molecules of the compound (when the isotopic content is accurately known), allowing complete mixing to occur, and continuing as in direct isotope dilution analysis. This method can be used only where the mixture contains, as the unknown, a substance that is already isotopically labeled with a known amount of label.[1]

The substance being analyzed is diluted with the desired quantity of unlabeled substance, accurately measured, or, where a derivative is made, with unlabeled derivative. Important advantages are realized from this procedure where it is possible to use it. Of prime importance is the fact that microgram concentrations and less can be accurately analyzed. Sufficient unlabeled material may be added to insure ease of isolation and purification of the material after mixing, since the more unlabeled material that is added, the more accurate are the results. The only limitation on the amount of unlabeled material which may be added is the original isotopic concentration of the labeled material. Dilution must not be carried out to such an extent that the isotopic concentration can no longer be measured. The extent of dilution can be easily calculated from the original isotopic concentration, which is known. Another advantage of the method is the fact that the material added in the dilution step need not be labeled. Where a labeled derivative is used to make the compound measured of known isotopic content, a single labeled reagent may suffice for many different determinations.

The purity of the isolated product is of great importance especially if more than one substance is labeled with the same isotope, and extreme care must be taken on this point. The problem is less acute if only one substance in the mixture is labeled. In short, the experiment may be run under normal conditions, with no radioactivity present, and the radioactive material is not added until the final stage of analysis.

In inverse isotope dilution analysis, the calculations are based on the isotopic concentration before and after mixing and on the weight of unlabeled material added. The following equation is applicable:

$$G_1 = G_2 \left(\frac{\text{Sp. Ac.}_f}{\text{Sp. Ac.}_i - \text{Sp. Ac.}_f} \right)$$

where G_1 = weight of labeled material (unknown originally present) being analyzed, G_2 = weight of unlabeled material added, Sp. Ac._i = specific activity of G_1, and Sp. Ac._f = specific activity of the isolated sample ($G_1 + G_2$).

A similar equation is used where atom % excess values are obtained with stable isotopes. If high isotopic concentrations are used, a correction must be made as described for direct isotope dilution analysis.

The criteria necessary for indirect isotope dilution analysis are essentially those listed in Table I, except that milligram or greater quantities are not required and isotopic labeled reagent is not needed. When an isotopic labeled derivative is prepared, isotopic labeled reagent must be available. The

[1] In the usual method the experiment is conducted with labeled material and the unlabeled material is added directly as the first step of analysis.

An alternative procedure is to conduct the experiment with unlabeled material. As a preliminary step to the analysis, the unlabeled material is then reacted quantitatively with a labeled reagent, producing a labeled derivative. Knowing the isotopic concentration of the reagent, the isotopic concentration of the derivative may be calculated. The actual analysis results in a determination of the amount of derivative which was formed. The amount of material originally present, which reacted quantitatively with the reagent, is then readily calculated.

isotopic concentration of the reagent must be high enough to withstand the dilution required.

An additional criterion is that the reaction between the labeled reagent and the compound must be quantitative.

This method has all of the advantages listed in Table II; in addition, it can be used for the determination of trace quantities (in many cases less than 10^{-9} Gm.), and quantities of carrier (unlabeled derivative) may be added with only the limitation of the dilution factor of the original isotopic concentration considered. In addition, a single isotopic labeled reagent may suffice for the analysis of many compounds.

The big disadvantage to this method where it can be used is the high purity necessary for the isolated sample.

Double Isotope Dilution Analysis.—In both direct and indirect isotope dilution methods of analysis, specific activities must be known. In a modification by Block and Anker (4) analysis is made of a compound which arises by such a process that its specific activity cannot be determined.

When the amounts of an intermediate compound (e. g., a metabolite, Z) which are present are too small to permit isolation and purification so that the specific activity (Sp. Ac.$_{iz}$) may be determined, two aliquots may be taken and a different amount of carrier (G_2, G_3) added to each. Equations 1 and 2 apply:

$$Z = G_2 \left(\frac{\text{Sp. Ac.}_{f\text{-}2}}{\text{Sp. Ac.}_{iz} - \text{Sp. Ac.}_{f\text{-}2}} \right) \qquad (1)$$

$$Z = G_3 \left(\frac{\text{Sp. Ac.}_{f\text{-}3}}{\text{Sp. Ac.}_{iz} - \text{Sp. Ac.}_{f\text{-}3}} \right) \qquad (2)$$

where Z = unknown amount of metabolite, Sp. Ac.$_{iz}$ = unknown specific activity of metabolite, G_2 = amount of carrier added, G_3 = amount of carrier added, Sp. Ac.$_{f\text{-}2}$ = final specific activity of G_2 aliquot material, and Sp. Ac.$_{f\text{-}3}$ = final specific activity of G_3 aliquot material.

With two equations, one may solve for the two unknowns:

$$Z = \frac{\text{Sp. Ac.}_{f\text{-}2}\, G_2 - \text{Sp. Ac.}_{f\text{-}3}\, G_3}{\text{Sp. Ac.}_{f\text{-}3} - \text{Sp. Ac.}_{f\text{-}2}}$$

$$\text{Sp. Ac.}_{iz} = \frac{(\text{Sp. Ac.}_{f\text{-}2})(\text{Sp. Ac.}_{f\text{-}3})(G_2 - G_3)}{(\text{Sp. Ac.}_{f\text{-}2})G_3 - (\text{Sp. Ac.}_{f\text{-}3})G_2}$$

Since the final specific activity may be determined in each case (Sp. Ac.$_{f\text{-}2}$, Sp. Ac.$_{f\text{-}3}$) and the amounts of carrier (G_2, G_3) are known, both the amount of metabolite present (Z) and its specific activity (Sp. Ac.$_{iz}$) may be calculated. Although the inherent error is rather large, it is certainly not too large for many metabolic studies where biological variation is also great.

DISCUSSION

Isotope dilution analysis is a recent modification of tracer analysis and is particularly useful when the substance analyzed cannot be quantitatively separated from a mixture or when the analytical problem cannot be readily solved by other means. The method is not to be considered simple in nature, since it requires specialized skills by the worker and may be quite complex and tedious. The success of the method is determined by the type of application and the thoroughness of operation. Like all other analytical procedures, the method cannot be used indiscriminately for solving a variety of analytical problems. On the other hand, the value of the method where utilization is possible and justified cannot be denied. The isotope dilution procedure of analysis has without question already established itself as a new and powerful analytical tool which will grow in scope of application, providing another method for solving the numerous problems of the analytical chemist.

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The Quantitative Recovery and Determination of Strychnine from Toxicological Samples*

By WILLIAM ROTH,† LOUIS ARRIGONI,‡ and LOUIS FISCHER§

A method is presented for the isolation and quantitative determination of strychnine from toxicological samples. The recovery of the alkaloid is accomplished by treating the sample with pancreatin, followed by a simplified extraction procedure on filtered aliquots of the diluted sample. The residue of extracted strychnine is reduced with zinc and hydrochloric acid and treated with sodium nitrite. The red color developed is estimated colorimetrically at 530 mμ. Determination on samples containing from 0.1 to 1.0 mg. give an average recovery of 98.9%, compared to 84.6% achieved by the Haines modification of the Dragendorff method.

THE toxic character and availability of strychnine in many forms necessitates a rapid and accurate method for the recovery and quantitative determination of the alkaloid from toxicological samples.

Stas (1) developed a method for the recovery of alkaloids from animal tissues by extraction with acidulated alcohol followed by evaporation of the alcoholic extract. The residue was taken up in water and the aqueous solution was made alkaline with sodium bicarbonate and extracted with ether. This method was modified by various workers (2–5), and at present the modifications are widely used (5–7). The drawbacks of the method are incomplete extraction with alcohol, formation of emulsions when extracting with immiscible solvents, and the amount of time required.

Several workers (8–13) have reported success with preliminary digestion of the sample with proteolytic enzymes before extraction with immiscible solvents.

Malaquin (14) reported a test for strychnine in which a red color was formed by reducing the alkaloid with zinc and hydrochloric acid and adding concentrated sulfuric acid. Denigés (15), in his study of Malaquin's reaction, found that the color developed from traces of nitric and nitrous acid in the reagents, and suggested the reaction as a colorimetric method for the estimation of strychnine or nitrates. Francois (16) reported a quantitative method for determining strychnine in pharmacopoeial products with zinc amalgam and hydrochloric acid to reduce the alkaloid, and Snell (17) outlined a similar procedure.

In the present investigation, simulated toxicological samples were subjected to enzymatic digestion followed by an extraction procedure that eliminated troublesome emulsions. The alkaloid was then determined quantitatively by an application of the color reaction.

EXPERIMENTAL

Preparation of Simulated Toxicological Samples.—Fresh beef liver was ground in a food chopper, and 10-Gm. samples were transferred to beakers. A measured amount of strychnine solution of known concentration was added to each sample.

Selection of Enzyme.—Pancreatin was used as the enzyme because of its availability, its content of amylolytic and lipolytic enzymes, and the effectiveness of its proteolytic enzymes. Sodium carbonate was used as a buffer to maintain a *p*H of 8–9 during the digestion.

Extraction Procedure.—The following procedure was developed to avoid emulsion formation. After digestion with pancreatin the sample was adjusted to a definite volume with dilute acetic acid. An excess of acetic acid was added to assure solution of the free alkaloid and to aid in filtration. After evaporation of an aliquot of the filtrate, the residue was taken up in a minimum amount of 15% sodium carbonate solution and extracted by stirring with benzene. A sodium carbonate solution of 15% provided a sufficiently high electrolyte concentration to prevent the formation of emulsions. If higher concentrations of sodium carbonate were used, some of the salt would separate during the extraction, forming a scum with organic material. Benzene was selected as a solvent in preference to chloroform because it had less tendency to form emulsions and to dissolve protein impurities.

Colorimetric Procedure.—Strychnine was reduced with zinc and hydrochloric acid, and after decanting the acid solution from the zinc, the color was developed by the addition or 0.1% sodium nitrite solution. For full color development, it was necessary to carry out the reduction for twenty minutes, adding one-half of the acid at the start and the other half after ten minutes. The solution exhibited maximum absorption at 530 mμ. The color developed immediately and was stable for at least three hours. Standards were reproducible to an average of 1% in transmission and the color formed followed Beer's Law in the range used. All absorption measurements were made with a Beckman Model DU spectrophotometer. Distilled water was used as a blank, since the preliminary investigation of the colorimetric procedure showed that an extract

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prepared by the proposed method, using liver samples without strychnine, did not exhibit absorption at the chosen wave length.

Method.—A series of ten 800-ml. tall form beakers containing 10 Gm. of ground liver and amounts of strychnine between 0.1 and 1.0 mg. (Table I) were prepared. To each sample was added 1 Gm. of pancreatin U. S. P., 0.4 Gm. of sodium carbonate, and 20 ml. of distilled water. The mixture was stirred with an electric mixer, and the stirrer and sides of the beaker were washed with a small amount of water. The mixture was then placed in an incubator at 37° for twenty-four hours. After the first twelve hours, the *p*H was adjusted to 8 with 0.5 *N* sodium hydroxide. At the end of the incubation period, the sample was transferred to a 250-ml. volumetric flask, the beaker was washed with water until the flask was about two-thirds full, and was then washed with two 30-ml. portions of 3% acetic acid. Care was taken that the solution in the flask was acid at this point. A few drops of ethyl ether were added to the flask to suppress the foam, and the contents of the flask were made to volume, mixed, and filtered through a No. 42 Whatman filter paper.

An aliquot of 10–50 ml. of the filtrate (equivalent to 0.02 to 0.04 mg. of strychnine) was transferred to a small casserole and evaporated on a steam bath. Care was taken not to overheat the residue and thus decrease its solubility. After cooling, the residue was taken up in 0.5 to 1.0 ml. of 15% sodium carbonate solution. Ten milliliters of benzene was added to the casserole, vigorously stirred with the aqueous layer for three to four minutes, and decanted into a 50-ml. beaker. The benzene extraction was repeated three times. A few drops of the sodium carbonate solution were added occasionally to keep the aqueous phase fluid. The combined benzene extracts were evaporated to dryness on a steam bath.

Two milliliters of distilled water, 2 ml. of hydrochloric acid, and 2 Gm. of 30 mesh granular zinc were added to the beaker containing the strychnine residue. The beaker was covered with a watch glass and placed on the steam bath for ten minutes. An additional 2 ml. of hydrochloric acid was added and the reduction was allowed to continue for an additional ten minutes. The watch glass was rinsed with 0.5 ml. of water, and the cooled solution was decanted from the unreacted zinc into a 10-ml. volumetric flask. The beaker and zinc were washed three times with 0.5-ml. portions of water, and the washings were added to the flask. Hydrochloric acid (0.5 ml.) and one drop of 0.1% sodium nitrite solution were added to the flask and the contents were made to volume. The solution was mixed and transferred to a 1-cm. cell and the transmission was read at 530 mμ. The concentration of strychnine was calculated from a standard curve prepared as outlined below. The results from his method on ten levels of strychnine are shown in Table I. Duplicate samples were run at each level, and two aliquots from each sample were extracted.

TABLE I.—RECOVERY OF STRYCHNINE BY THE PROPOSED METHOD

Mg. Strychnine Added	Av. Mg. Strychnine Recovered
0.100	0.088
0.200	0.182
0.300	0.282
0.400	0.416
0.500	0.559
0.600	0.546
0.700	0.734
0.800	0.820
0.900	0.920
1.000	0.998

The standard curve was prepared by measuring a series of strychnine standards (0.01 to 0.06 mg.) into 50-ml. beakers and adding enough water to adjust the initial volume to 2 ml. Acid and zinc were then added and the color was developed and measured. The logarithm of the per cent transmission was plotted against the concentration.

Evaluation of the Method.—In order to compare the proposed method with that now in use, 5 samples were extracted by the Haines modification of the Dragendorff method (5). Samples were prepared as described in the proposed method and chloroform was used as the immiscible solvent. The extracted strychnine was dissolved in hydrochloric acid (1:1) and diluted with the same acid to a volume that would contain approximately 0.02 mg. of strychnine in each 4 ml. The strychnine was determined in 4-ml. aliquots by the colorimetric procedure previously outlined. The results are reported in Table II. The strychnine recovered represents the average of two aliquots for each sample.

TABLE II.—RECOVERY OF STRYCHNINE BY THE HAINES MODIFICATION OF THE DRAGENDORFF METHOD

Mg. Strychnine Added	Av. Mg. Strychnine Recovered
0.100	0.089
0.300	0.264
0.500	0.434
0.700	0.630
0.900	0.622

SUMMARY

1. A method for the determination of strychnine in toxicological samples is outlined, using pancreatin to digest the tissue material and a simplified method of extraction with benzene as a solvent. The recovered strychnine is then determined by a modification of an existing colorimetric procedure.

2 Determinations made on samples containing from 0.1 to 1.0 mg. gave an average recovery of 98.9 per cent. In comparison, the Haines modification of the Dragendorff method gave an average recovery of 84.6 per cent.

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Sulfonamide Separations Based on Ion-Exchange Chromatography in Combination with Radioactive Techniques*,†

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A method is presented for the quantitative determination of sulfonamides based on the differences in their distribution coefficients. It appears to be suitable for the assay of sulfonamides and their derivatives in pure form as well as in pharmaceutical preparations. The procedure consists essentially in the adsorption of the sulfonamides from an aqueous acidic solution on a column of ion-exchange resin. Finally, the individual sulfonamides are chromatographically eluted and measured quantitatively using the isotope dilution technique.

THE DISCOVERY in 1935, by Adams and Holmes (1), that phenol-formaldehyde resins exhibited base-exchange properties aroused considerable interest in the development and industrial applications of synthetic resins as ion-exchangers, particularly in the pharmaceutical industry.

To the present time, the sulfonamides appear to be the only major group of pharmaceutical compounds which have not been subjected to investigation using ion-exchange resins. Many types of procedures have been proposed for the identification, separation, and estimation of sulfonamides. The majority of these methods either require time-consuming chemical manipulations or are valid for only a single sulfonamide. Thus a simple, clear-cut method of separating and quantitatively estimating sulfonamides, singly and in groups, in medicinals, reaction mixtures, biological fluids, etc., should be of great advantage. Ion-exchange resins appear to offer an ideal solution to this problem. The purpose of this work was to determine a means by which sulfonamides could be separated from solution and analyzed by the use of ion-exchange resins in combination with radioactive tracer techniques.

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† The isotope used in this research was granted on an allocation from the United States Atomic Energy Commission.
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EXPERIMENTAL

Resins and Reagents.—The following materials were used:

(a) Amberlites IR-120, IRC-50, IR-4B, and IRA-400; Dowex-50; Permutit-Q; and Catex 27 and 55.[1]

(b) U. S. P. XIV and N. F. IX sulfonamides (sulfanilamide, sulfadiazine, sulfathiazole, sulfaguanidine, sulfamerazine, and sulfapyridine).

(c) Sulfur-35 labeled sulfanilamide and sulfadiazine and their acetyl derivatives.[2]

Apparatus.—Three types of ion-exchange resin columns were utilized in this investigation. A semimicro column (3) was employed for trial runs. For single sulfonamide separations, a medium-length column was used, consisting of a 30 x 1.8-cm. (i. d.) Pyrex tube fitted with a one-hole stopper containing a 7-cm. glass tube which was connected to

[1] The authors are indebted to the manufacturers of these resins for the samples which they supplied.
[2] Synthesized by P. J. Byrne, Jr., A. A. Alberts, and J. E. Christian (2).

5 cm. of Tygon tubing held by a screw clamp to regulate the flow rate. A long column for chromatographic separations was prepared in an identical manner except that a 60 x 1.3-cm. (i. d.) Pyrex tube was used.

For making all determinations of radioactivity, a device was employed consisting of a Tracerlab 1.6 mg./sq. cm. thick, end-window G-M tube mounted in a Technical Associates Model AL14A lead shield and connected to a Model SC2A Tracerlab "64" Scaler.

PROCEDURE

Radiochemical Analysis.—The procedure adopted consisted of pipetting a 1-ml. sample directly into a glass cup (prepared by cutting off the bottom of a 25-ml. glass ampul 9 mm. from the base), drying under infrared lamps, and counting on the top shelf of the G-M tube holder in the lead shield. Three samples, each counted in two positions, were utilized in order to achieve statistically accurate results. Corrections for background, decay, coincidence counts, and variation in counter efficiency were applied, where necessary.

Distribution Coefficient Determinations.—From a knowledge of the value of the distribution coefficients (K_d) in a particular investigation, several facts become readily apparent; among these is the fact that the ion possessing the largest K_d value has the greatest affinity for the resin and will be eluted last from a column of resin containing similar ions. Assuming that the mass action law may be applied to the reaction under investigation, then:

$$K_d = M_s/M_l \times v/w$$

where M_s and M_l are the fractions of the cation in the resin and liquid phases, respectively, and V is the volume (ml.) of solution added to W grams of resin.

The K_d was determined as follows: 10 or 25 ml. of a standardized solution of the S-35 labeled sulfonamide:HCl was pipetted into 30-ml. serum bottles containing various accurately weighed amounts of the cation-exchanger (thoroughly converted to the free acid form, washed, and dried to constant weight at 105°). The bottles were then sealed and placed in a shaker where they were allowed to equilibrate for seventy-two hours. One milliliter samples were removed from each bottle and counted by the glass cup technique. Table I shows the K_d values for several resin: sulfonamide systems. The ratio of average K_d values for any given resin: sulfonamide system provides a numerical index called the separation factor. When this factor is 1.2 or greater, the two cations may be separated by chromatographic elution techniques. Table I shows that the separation factor for all of the various resin: sulfonamide systems under consideration is greater than 1.2. Therefore the two sulfonamides may be separated by chromatographic elution techniques and sulfanilamide will be eluted before sulfadiazine.

The Removal and Recovery of Various Sulfonamides from Their Respective Solutions.—All official sulfonamides contain a free amino group capable of being exchanged with another cation derived from an ion-exchange resin. Since the sulfonamide:HCl solution will be acidic, a sulfonic or carboxylic acid resin and not a phenolic resin appears to be the most suitable for these separations.

The general scheme of carrying out these ion-exchange experiments in order to study the columnar behavior of single sulfonamides and their acetyl derivatives is as follows: the ion-exchange resin is wet screen-graded and the 10–30 mesh size is packed into a medium-length column. The resin is then backwashed with at least 500 ml. of distilled water to remove the fines and classify the resin (after each experiment and before subsequent backwashings, 10 resin volumes of 5% EtOH are passed through the column to remove any adsorbed organic material). At least twice the theoretical amount of acid (10% HCl or 5% H_2SO_4) necessary for complete regeneration, determined from resin data sheets obtained from the manufacturer, is used as the regenerant. The resin is now washed with distilled water until the effluent is completely free of the regenerant as shown by the absence of a precipitate upon the addition of a 5% $AgNO_3$ solution for HCl (or 1% $Ba(OH)_2$ solution for H_2SO_4). A known amount of

TABLE I.—EFFECT OF RESIN WEIGHT ON THE K_d VALUES OF SEVERAL RESIN-SULFONAMIDE:HCl SYSTEMS

	Sulfanilamide:HCl				Sulfadiazine:HCl				Ratios of Av. K_d Values
Resin	Resin Wt. Gm.	Sulfa Concn. $\times 10^{-3}$ M	Volume, Ml.	K_d	Resin Wt. Gm.	Sulfa Concn. $\times 10^{-3}$ M	Volume, Ml.	K_d	
A. Amberlite IR-120 (H) A.G.	0.2368	9.5	10	21.3	0.3859	3.5	10	57.7	2.45
	0.5493	9.5	10	21.2	0.5333	3.5	10	57.6	
	0.7191	9.5	10	22.0	0.6767	3.5	10	57.7	
	0.7385	9.5	10	20.5	1.5208	3.5	10	58.4	
B. Dowex-50	0.1062	1.5	25	404.8	0.1372	1.1	25	1282.6	3.03
	0.1597	1.5	25	436.8	0.1788	1.1	25	1297.2	
	0.2024	1.5	25	407.5	0.3374	1.1	25	1295.0	
C. Permutit-Q	0.1274	2.4	25	108.3	0.2162	.51	25	381.4	3.42
	0.2989	2.4	25	107.0	0.2825	.51	25	356.5	
	0.4687	2.4	25	111.9	0.4393	.51	25	381.1	
	0.6303	2.4	25	108.5	0.7899	.51	25	381.2	
D. Catex-27	0.1953	2.2	25	117.4	0.2041	.24	25	1488.4	14.27
	0.3716	2.2	25	120.9	0.4158	.24	25	1857.0	
	0.5644	2.2	25	137.0	0.8192	.24	25	1894.0	
	0.6593	2.2	25	144.0	0.8670	.24	25	2217.6	
E. Catex-55	0.1247	1.4	25	220.4	0.0919	.55	25	1904.0	12.22
	0.1930	1.4	25	246.0	0.2978	.55	25	3758.7	
	0.2154	1.4	25	255.2	0.3203	.55	25	3892.2	
	0.5667	1.4	25	396.9	0.4206	.55	25	3967.9	

TABLE II.—RESULTS OF SULFONAMIDE SEPARATIONS USING AMBERLITE IR-120(H) A. G.[a]

Sulfonamide	Sulfa Added, Gm.[b]	Resin Bed Depth,[c] Cm.	Resin Mesh Size	Aq. Effluent Vol. Ml.[e]	Acidic Effluent Vol. Ml.[f,g]	Leakage, %	Exchange, %
Sulfanilamide	0.4930	30	10–30	100	800	0.00	98.40
	0.9860	30	10–30	200	900	0.00	99.14
	0.9860	30	10–30	200	800	0.00	99.14
	0.0408	21	10–30	200	900	0.00	99.20
N^4-Acetylsulfanilamide	0.0854	..[d]	40–60	300	200	99.70	0.80
	0.0648	30	10–30	500	500	100.00	0.00
Sulfadiazine	0.0898	30	10–30	200	2,000	0.00	100.00
	0.0187	21	10–30	200	1,700	0.32	99.20
N^4-Acetylsulfadiazine	0.0292	..[d]	40–60	300	200	98.20	1.60
Sulfaguanidine	0.4855	30	10–30	500	3,500	0.00	99.60
	0.0739	30	10–30	500	2,000	0.00	99.90
Sulfamerazine	0.5150	30	10–30	300	2,800	0.00	100.30
	0.5150	30	10–30	300	2,800	0.00	100.60
Sulfathiazole	0.2470	30	10–30	300	2,200	0.00	99.95
	0.2470	30	10–30	300	2,400	0.00	100.02
Sulfapyridine	1.2470	30	10–30	300	2,500	0.00	99.97
	1.2470	30	10–30	300	2,500	0.00	100.01

[a] The flow rate was held constant at one drop per second.
[b] These weights represent the amount of sulfa in an aliquot (10 or 25 ml.) of a solution prepared by dissolving the sulfa in sufficient HCl (1–4 *N*) and adding distilled water to a desirable volume (100–200 ml.). In the case of the acetyl derivative, the solvent consisted of 2 parts of EtOH, 1 part 4 *N* HCl, and 1 part HOH.
[c] Pyrex columns (60 x 1.8 cm. i. d.) were used.
[d] Semimicro ion-exchange columns (3).
[e] Total volume of aqueous effluent obtained by passing the sulfonamide solution (10–25 ml.) through the column and then adding sufficient distilled water to insure thorough contact of the sulfonamide with the resin.
[f] 4 *N* HCl was elutriant in all cases except runs using 0.4930 Gm. of sulfanilamide (2 *N* HCl) and 0.9860 Gm. of sulfanilamide with a 900-ml. acidic effluent volume (3 *N* HCl).
[g] Volume of effluent required before all the sulfonamide was eluted from the resin as shown by analysis. This was collected in 100-ml. portions except in experiments using sulfaguanidine and 0.648 Gm. of N^4-acetylsulfanilamide (500-ml. portions); 25-ml. portions of the aliquots were analyzed by the U. S. P. $NaNO_2$ assay procedure with the exceptions of runs using 0.0403 Gm. of sulfanilamide, 0.0854 Gm. of N^4-acetylsulfanilamide, 0.0187 Gm. of sulfadiazine, and N^4-acetylsulfadiazine which were analyzed by taking 1-ml. portions and determining the radioactivity in circular glass cups (diameter, 22 mm.; depth, 8 mm.) after drying under infrared light.

the sulfonamide:HCl in aqueous acidic solution is now added to the column, the effluent flow rate is carefully adjusted and, using sufficient distilled water to supply the volume, samples (100–500 ml.) are collected in volumetric flasks (by analyzing these samples, the leakage is determined). The column is now eluted using the proper elutriant and samples (100–500 ml.) are collected until all of the sulfonamide is removed from the column (analysis of these latter samples shows the percentage of exchange).

Table II shows details of the final procedures designed to effect the removal and recovery of single sulfonamides from their respective separate solutions by means of Amberlite IR-120(H)A.G. All of the procedures listed employ the general method previously described. The sulfonamide solutions used, with the exception of the acetyl compounds which were dissolved in a solvent consisting of 2 parts EtOH, 1 part 4 *N* HCl, and 1 part HOH, are aqueous acidic solutions in the sense that they contain sufficient HCl to dissolve the sulfonamide and form the hydrochloride. The results presented in Table II show that Amberlite IR-120(H)A.G. can quantitatively remove the sulfonamides from solution and also quantitatively release them by use of a suitable elutriant. The acetyl derivatives are essentially unaffected by the resin since leakages of approximately 100% are observed.

Separation of Sulfanilamide and N^4-Acetylsulfanilamide.—From the results shown in Table II it is apparent that Amberlite IR-120(H)A.G. quantitatively removes sulfanilamide from solution and allows its acetyl derivative to pass through to give 100% leakage. This fact suggested a means for separating sulfanilamide from its acetyl derivative. A satisfactory procedure was worked out as follows: 25 ml. of an aqueous acidic solution containing 0.105 Gm. of sulfanilamide and 0.0679 Gm. of N^4-acetylsulfanilamide was passed through a column of Amberlite IR-120(H)A.G. (resin bed depth of 30 x 1.8 cm.). Sufficient distilled water was added to enable the collection of a 500-ml. sample, and a 25-ml. aliquot was analyzed both qualitatively and quantitatively for sulfanilamide and the acetyl compound. A leakage of 0.0% sulfanilamide and 100% of the acetyl derivative was obtained. One thousand milliliters of 4 *N* HCl elutriant was then added and the effluent was quantitatively analyzed for sulfanilamide, using the U. S. P. sodium nitrite assay. This analysis showed that 99.07% exchange and recovery of the sulfanilamide had occurred. Results of a similar experiment showed 100% leakage of the acetyl derivative and 0.0% leakage of sulfanilamide followed by a 99.6% exchange and recovery of the sulfanilamide on elution.

Separation of Sulfanilamide and Sulfadiazine.—Utilizing the information presented in Table II for the separation and elution of separate solutions of sulfanilamide and sulfadiazine, attempts were made to separate these two from a common solution Results indicated that such a separation was possible by using 1 *N* or 2 *N* HCl elutriant instead of 3 *N* or 4 *N*, 40–60 mesh resin instead of 10–30 mesh, and a 60 x 1.3-cm. instead of the usual 30 x 1.8-cm. resin bed depth employed in the separation of single sulfonamides. The following procedure was found to be successful: 25 ml. of an aqueous, acidic solution containing sulfanilamide (0.2493 Gm.) and sulfadiazine (0.285 Gm.) was pipetted into a column con-

taining a 40–60 mesh, 60-cm. resin bed depth in a 120 x 1.3-cm. (i. d.) tube. By the addition of distilled water, three 100-ml. samples were collected. The column was eluted with 5,400 ml. of 1 *N* HCl and 100-ml. samples were collected. Analysis of 25-ml. aliquots of all samples by the U. S. P. sodium nitrite titrimetric procedure showed a leakage of 0.0% and an exchange and recovery of 99.86%. The elution curve gave two separate peaks, showing that there was a complete separation of the two compounds. According to the K_d values, sulfanilamide should be released first from the column, followed by sulfadiazine. This theory was substantiated by U. S. P. qualitative identification tests carried out on samples representing the peak concentrations of the elution curve. In order to check the reproducibility, an identical experiment was run using 25 ml. of a solution containing 0.2781 Gm. of sulfanilamide and 0.5020 Gm. of sulfadiazine. Results compared favorably with previous findings in that 0.0% leakage, 100.02% exchange, and a complete separation were noted.

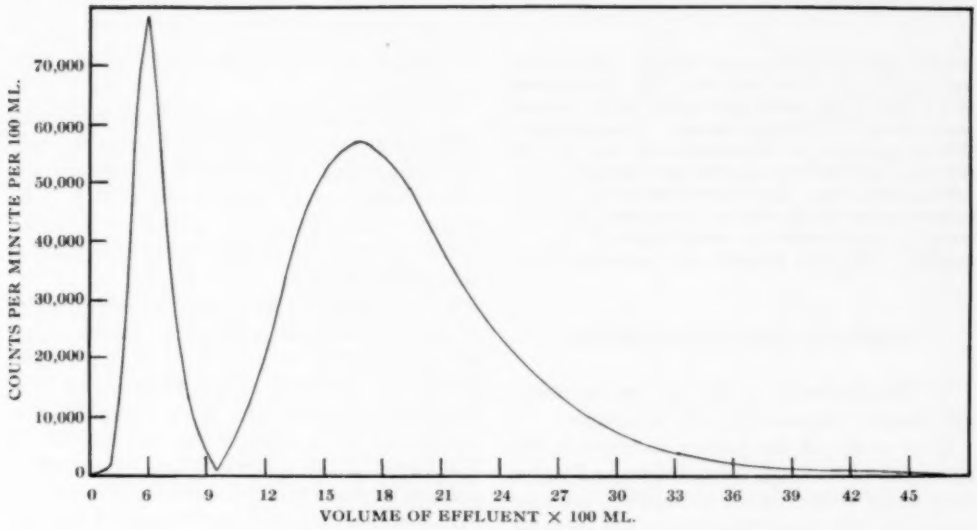

Figure 1.

The entire method of procedure described above was checked by using S-35 labeled sulfonamides in order to obtain a more accurate picture of the degree of separation. All experimental conditions utilized here were identical to those previously described with the following exceptions: (*a*) samples were analyzed by radioactive counting techniques; (*b*) 2 *N* HCl was used as the elutriant; (*c*) 50 ml. each of solutions of S-35 sulfanilamide (0.11%; specific activity, 3062 c. p. m./mg.) and S-35 sulfadiazine (0.12%; specific activity 10,679 c. p. m./mg.) were added to the resin column. Results, pictured graphically in Fig. 1, showed that a complete separation was effected, since 0.15% leakage and 100.06% total recovery were observed.

Analysis of Sulfanilamide and Sulfadiazine in a Typical Medicinal Dosage Form.—After the separation of sulfanilamide and sulfadiazine had been achieved, using a column of Amberlite IR-120(H) A.G., a method was developed for the quantitative determination of these two sulfonamides as they occur in a typical medicinal dosage form. The method of measurement was based on recently proposed isotope dilution techniques (4). The following is a brief discussion of a typical analysis by the method developed.

The ingredients in a typical tablet formulation were used in the following quantities which represent 8 tables (sulfanilamide, 2.0385 Gm.; sulfadiazine, 2.0138 Gm.; cornstarch, 0.6 Gm.; 5% gelatin-acacia solution, 1.6 ml.; talc, 0.04 Gm., and magnesium stearate, 0.04 Gm.). This mixture was accurately weighed and placed in a beaker containing 50 ml. of 2 *N* HCl. The resulting mixture was thoroughly stirred to dissolve the sulfonamides completely, and was filtered into a 200-ml. volumetric flask. The beaker and residue were rinsed with small successive portions of 2 *N* HCl. These rinsings were added to the flask and the total volume was diluted to the 200-ml. mark with distilled water. The analysis was carried out on a 25-ml. portion of this solution.

For the analysis, a solution of S-35 labeled sulfanilamide was prepared by dissolving 0.4616 Gm. of the compound in 15 ml. of 2 *N* HCl contained in a volumetric flask and adding distilled water up to the 100-ml. mark. Using the glass cup technique, the specific activity of the sulfanilamide, determined by taking a 1-ml. aliquot, drying, weighing, and counting, was found to be 7,478 c. p. m./mg. This procedure was repeated, using 0.1111 Gm. of S-35 labeled sulfadiazine (specific activity, 4,873 c. p. m./mg.). Twenty-five milliliters of each of these two solutions were mixed with the 25-ml. aliquot of the U. S. P. sulfonamide solution and then added to a 60 x 1.3-cm. (i. d.) resin bed depth of 40–60 mesh Amberlite IR-120(H)A.G.[3] Sufficient distilled water was added to provide for the collection of four 100-ml. samples, maintaining a flow rate of 1–2 drops/

[3] The column was regenerated as described previously.

SULFANILAMIDE ANALYSIS

Known weight of sulfanilamide (U. S. P. XIV) = 254.8 mg.
Known weight of sulfanilamide (Sulfur-35) = 115.4 mg.
S. A.[a] of sulfanilamide (S-35) = 34,000 c. p. m./4.6 mg.
= 7,478 c. p. m./mg.
S. A. of Sample No. 9 = 3,436.4 c. p. m./1.5 mg.
= 2,290 c. p. m./mg.

$$\text{Calculated weight of sulfanilamide (U. S. P. XIV)} = \frac{7,478}{2,290} - 1 \times 115.4$$

= 253.8 mg.
Per cent accuracy = 253.8/254.8 × 100 = 99.6%

SULFADIAZINE ANALYSIS

Known weight of sulfadiazine (U. S. P. XIV) = 251.7 mg.
Known weight of sulfadiazine (Sulfur-35) = 27.7 mg.
S. A. of sulfadiazine (S-35) = 5,410 c. p. m./1.1 mg.
= 4,873 c. p. m./mg.
S. A. of Sample No. 23 = 96.2 c. p. m./0.20 mg.
= 481 c. p. m./mg.

$$\text{Calculated weight of sulfadiazine (U. S. P. XIV)} = \frac{4,873}{481} - 1 \times 27.7$$

= 252.1 mg.
Per cent accuracy = 252.1/251.7 × 100 = 100.1%

[a] S. A. refers to specific activity (4).

second, in order to check for leakage. The leakage was found to be on the order of 0.1%. An elutriant of 1.5 *N* HCl was added and thirty 100-ml. samples were collected (1–2 drops/second). Two particular 100-ml. samples (No. 9-sulfanilamide and No. 23-sulfadiazine) which represented pure solutions of the sulfonamides, since they corresponded to the peak concentrations on the elution curve, were then analyzed by determining the specific activity of 1-ml. aliquots. The data obtained were as shown above.

SUMMARY AND CONCLUSIONS

1. The distribution coefficients for ten resin:-sulfonamide systems have been determined.

2. A study of the factors involved in the separation of six single sulfonamides and two of their acetyl derivatives from solution has been carried out with respect to flow-rate, ion-exchange resin type, resin bed depth, mesh size, type and strength of elutriant, per cent leakage and exchange, and degree and type of regeneration.

3. Ten per cent HCl has been shown to be more efficient as a resin regenerant than 5 per cent sulfuric acid.

4. Procedures have been worked out for the separation of sulfonamides from their acetyl derivatives.

5. A method of separating sulfanilamide from sulfadiazine has been developed which employs chromatographic elution from a column of resin. Measurements were made by the U. S. P. XIV sodium nitrite titrimetric procedure or radioactive counting techniques when S-35 labeled compounds are used.

6. A method of analyzing the sulfonamides present in medicinal preparations has been proposed, consisting of separation of the sulfonamides by chromatographic elution and their quantitative measurement by the isotope dilution technique. It is evident that the method is applicable to the qualitative and quantitative analysis of many types of sulfonamide mixtures, including the analysis of trace amounts in biological fluids.

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Chromatography of Vitamin B_1 and Choline*

By AUBIN HEYNDRICKX†,‡

The separation of vitamin B_1 and choline has been achieved by means of chromatography on starch. A qualitative determination is described; the quantitative tests are done by the usual methods.

THE combination of vitamin B_1 and choline is frequently used in pharmaceutical preparations. There are many problems connected with the attempt to develop an analytical method for the determination of these two substances, since they undergo nearly the same chemical reactions. They coprecipitate with phosphotungstic acid, silicotungstic acid, Reinecke salt, picric acid, mercuric chloride, picrolonic acid, Mayer's reagent, potassium hypoiodite, Millon reagent, cadmium chloride in alkaline solution, gold trichloride, etc.

Their solubilities in organic solvents are similar. They are insoluble in acetone, methyl alcohol, chloroform, and ether; nearly insoluble in isobutyl alcohol; and soluble in water and ethanol.

One method of separation makes use of the physicochemical properties of these two compounds. Martin and Synge introduced the use of starch columns in 1944, during their studies on paper chromatography, and Synge, also in 1944, used a starch column for the analysis of hydrolyzed gramicidin. This procedure was considered to have great possibilities for quantitative methods. Stein and Moore (1) developed the starch technique for quantitative use with a mixture of amino acids. The preparation of the column was explained and an automatic fraction collector was described (2, 3).

EXPERIMENTAL

Apparatus.—The small chromatograph tubes used in these experiments had a length of 40 cm. above the sintered glass plate and an inner diameter of 0.9 cm.

The large tubes (Fig. 1) had a length of 20 cm. above the sintered plate (D), and an inner diameter of 1.9 cm. Suction was effected with a water pump (A). A graduated cylinder (B) directly measured the volume of eluate, separate samples of which were collected in test tubes. A separatory funnel (C), containing the solvent, was connected at the top of the column. The system was placed in an air-conditioned room at 20 ± 1° C. The setup described was used only with the large tubes.

Preparation of the Starch Column.—Because of changes in atmospheric humidity, the water content of the starch was measured before the suspensions were prepared. A sample of starch was dried to constant weight at 110°.

A sample corresponding to 13.4 Gm. of anhydrous starch was suspended in 25 cc. of anhydrous *n*-butanol, to which an amount of water corresponding to 30% of the weight of dry starch was added and the mixture was stirred to obtain a uniform suspension, and was then poured down the side of the tube to avoid air bubbles. After the starch column had been prepared (30 cm. height for small columns, 15 cm. for the large ones), and the settlement had taken place (one to two hours), the supernatant liquid was removed with a pipette. A solvent, consisting of *n*-propyl alcohol and 0.1 *N* HCl in a 2:1 ratio, was added. About 250 cc. of this solvent was passed through the column, the wash being accelerated by suction. The process requires approximately ten and twenty-four hours for the large and small tubes, respectively. Caution must be exercised at all times to maintain the level of solution above the adsorbent, in order to prevent formation of air bubbles in the column. The rate of flow of the solvent was about 1.5 cc./hr. for the small columns (without suction). The rate of flow was influenced

Fig. 1.—Chromatography apparatus with suction.

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† The author wishes to acknowledge the cooperation of R. Ruyssen, of the University of Ghent, Belgium.
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by the type of starch, the water content, and the preparation of the column. In general, the water content should be decreased if the rate of flow is too rapid, and increased if the rate of flow is too slow.

Starting the Chromatogram.—The most favorable results are obtained with different concentrations of each compound. In general, vitamin B_1 is present in a much smaller amount, making separation easier. Choline and thiamine in powders and suspensions were extracted with a mixture of *n*-propyl alcohol and 0.1 *N* HCl in a 2:1 ratio, and were determined by the procedure described. Samples containing 500 mg. of choline and 0.1 mg. of thiamine on analysis yielded results which varied from the theoretical value no more than 3% for choline and 5% for thiamine.

Small Columns.—A mixture of 0.2 cc. of a 1% solution of thiamine HCl in the propyl alcohol-acid solvent and 0.2 cc. of a 1% solution of choline HCl in the same solvent was introduced into the column. A portion of 2 cc. of the solvent was then added from the separatory funnel in order to clean the walls of the tube. This procedure was repeated twice, and the solvent was then added to the column. The total eluate was collected in 0.5-cc. portions in separate tubes without the use of suction (Table I).

TABLE I.—SMALL COLUMNS

Product	Effluent, Cc.[a]	Column No.
Choline HCl	21–25	6
Choline HCl	20.5–24.5	7
Choline HCl	22–27	8
Thiamine HCl	35–40	6
Thiamine HCl	33.5–38	7
Thiamine HCl	36–41.5	8

[a] Fractions containing the compound.

Identification of the Material.—A drop of the eluate was placed upon filter paper and allowed to dry. A fresh solution containing 1% potassium ferrocyanide in water was then spread over the paper, followed by a second solution of 0.5% cobalt chloride in water. The presence of choline was indicated by the appearance of a green spot within a few seconds. The reaction is sensitive to 10 γ.

A second sample of filter paper, saturated with the eluate, was spread with a solution of 2 *N* NaOH, containing 5% of potassium ferricyanide (freshly prepared). A green fluorescence under U. V. light indicated the presence of thiamine. The reaction is sensitive to 0.5 γ. Evaporation of the unknown on the paper is necessary because of interference of the eluant.

Quantitative Tests.—The quantitative method described in the British Pharmacopoeia (4) was used for the estimation of vitamin B_1. Choline was determined spectrometrically (5). The fractions containing the products were evaporated on the water bath to eliminate the interference of the propyl alcohol.

Large Columns.—A column 15 cm. in height was prepared. Suction was applied to adjust the rate of flow to 25 cc./hr. The same size sample was used as in the small column technique. The total eluate was collected in 2-cc. portions in separate test tubes, and choline and thiamine were identified as described above (Table II). At the separation point, 0.5-cc. fractions were collected. Quantitative tests were made in the same manner.

TABLE II.—LARGE COLUMNS

Product	Effluent, Cc.[a]	Column No.
Choline HCl	61–82	2
Choline HCl	59–80.5	3
Choline HCl	63–85	9
Thiamine HCl	83–108	2
Thiamine HCl	81–109	3
Thiamine HCl	86–112	9

[a] Fractions containing the compound.

SUMMARY

By means of chromatography on starch, a quantitative separation of vitamin B_1 and choline can be made. The separation can be made both rapidly and efficiently by the large column method, which gives an interference of 1 cc. between the two peaks. Results are reliable up to 3 per cent.

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The Polarographic Determination of Riboflavin*,[1]

By W. J. SEAGERS

Polarographic assay procedures have been developed for the determination of riboflavin (Vitamin B_2) in riboflavin U. S. P., partially purified riboflavin, solubilized riboflavin, and vitamin mixtures containing riboflavin. Comparisons are made between the results using this assay method and those with the usual fluorometric assay method on production samples.

COLORIMETRIC, fluorometric, and microbiological methods have been used for the determination of riboflavin (vitamin B_2). A polarographic method has been suggested (1) for the assay of riboflavin in yeast but details of the proposed method or results of assays were not given. The present paper reports the procedures used for the assay of various products containing riboflavin, the assay results obtained, and a comparison of these data with those obtained using the fluorometric technique (2). Over the *p*H range 1 to 12, riboflavin exhibits a well-defined wave (1, 3) which is suitable for assay purposes. In the following procedures the *p*H of the solution polarographed depends on the extraction procedure used and the subsequent treatment. The extractive procedure used will depend on the material being assayed but, in general, three methods have been used which are described in detail.

Because the solution obtained on extraction of a vitamin mixture is of unknown composition, a standard addition method of analysis was used. For the sake of consistency, this same method was used for the other groups of materials.

EXPERIMENTAL

Materials.—The preparations assayed were regular production lots of riboflavin U. S. P., partially purified riboflavin, vitamin mixtures, and solubilized riboflavin. All chemicals were reagent grade and were used without further purification. Riboflavin reference standard U. S. P., dried at 100° for three hours, was used as a standard for all procedures.

Apparatus.—The polarographic cell was a 35 x 50-mm. weighing bottle provided with a four-hole rubber stopper in which were inserted a nitrogen inlet tube, the dropping-mercury electrode, a lead for contact with the mercury anode, and a thermometer. A standard dropping-mercury electrode (4) was used, care being taken that the height of the mercury head remained constant. In all cases, tank nitrogen, purged of oxygen by passage through an alkaline solution of pyrogallol, was bubbled through the cell solution for five minutes in order to remove oxygen. A Leeds and Northrup Electrochemograph Type E was used to record the polarograms.

Procedure.—The materials assayed can be divided into three groups containing those preparations which could be assayed by a common procedure.

Group A included riboflavin U. S. P. and partially purified riboflavin since both compounds may be assayed directly. A sample of 20–30 mg. of the material was accurately weighed and transferred into a 50-ml. volumetric flask, and 5 ml. of 0.4 *M* sodium hydroxide was added. The flask was swirled until solution was complete, and 20 ml. of aqueous buffer, 0.2 *M* with respect to acetic, boric, and phosphoric acids and 1.0 *M* with respect to potassium chloride, was then added. The solution was made up to volume with water and thoroughly mixed. A sample of 20–30 mg. of dried riboflavin reference standard U. S. P. was treated in the same manner. The solutions (*p*H about 2.8) were placed in the polarographic cell in turn, purged with nitrogen for five minutes, and polarographed over the potential range of 0 to −1 v. The diffusion currents were calculated in microamperes and the percentage of riboflavin was calculated from the following formula:

$$\text{Per cent Riboflavin} = \frac{i_d \text{ sample } (\mu\text{a.})}{i_d \text{ std. } (\mu\text{a.})} \times \frac{\text{mg. of std.}}{\text{mg. of sample}} \times 100$$

One standard was polarographed for each four determinations in order to correct for any changes in room temperature since all formulas used are based on the assumption that the temperature and the mercury head were constant. The results obtained with this procedure on representative samples are shown in Table I.

TABLE I.—A COMPARISON OF THE POLAROGRAPHIC AND FLUOROMETRIC ASSAY METHODS FOR RIBOFLAVIN IN GROUP A MATERIALS

Sample	Polarographic Assay, %	Fluorometric Assay, %
U. S. P. A	98.0; 97.2	99.5
U. S. P. B	99.2; 98.0	99.3
Partially purified A	91.6; 93.2	92.1
Partially purified B	89.2; 92.2	91.7

Group B included only solubilized riboflavin since the criterion of effective riboflavin content in this preparation is its solubility in water. A sample of 500–600 mg. of material was accurately weighed into a 100-ml. volumetric flask and diluted to volume with water; 10.0-ml. aliquots were pipetted

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[1] After this paper had been submitted for publication, a Paper by Fowler, S., and Kaye, R. C., *J. Pharm. Pharmacol.*, 4, 758(1952), described the polarographic determination of riboflavin in tablets and solution.

into two 50-ml. G. S. Erlenmeyer flasks. To one flask was added 10.0 ml. of a solution of one part 0.4 *M* sodium hydroxide to four parts of aqueous buffer (*cf.* Group A). To the other flask was added 10.0 ml. of standard, prepared by accurately weighing about 20 mg. of dried riboflavin reference standard U. S. P., adding 10 ml. of 0.4 *M* sodium hydroxide, swirling to dissolve, and diluting to 50.0 ml. with aqueous buffer. Each solution (*p*H about 2.8) was placed in the polarographic cell, purged with nitrogen for five minutes, and polarographed over the potential range 0 to −1 v. The diffusion currents were calculated in microamperes and the appropriate values were substituted in the following formula:

$$\text{Per cent riboflavin} = \frac{i_a\ (\mu a)}{i_b - i_a\ (\mu a)} \times \frac{\text{mg. of std. weighed}}{5} \times \frac{100}{\text{mg. of sample}}$$

where i_a is the diffusion current for the sample and i_b is the diffusion current for the sample plus standard. The results obtained using this procedure on representative samples compared with results of the fluorometric assay are shown in Table II.

TABLE II.—A COMPARISON OF THE POLAROGRAPHIC AND FLUOROMETRIC METHODS FOR RIBOFLAVIN IN GROUP B MATERIAL

Lot No.	Polarographic Assay, %	Fluorometric Assay, %
1	8.27	8.09; 8.29
2	9.50; 9.43	9.17; 9.35

Group C included vitamin mixtures containing riboflavin, one or more diluents, and, in some cases, one or more other vitamins. The sample size used in this group will depend on the amount of riboflavin present per ounce, i. e., 1,000 mg. per oz., 1-Gm. sample; 500 mg. per oz., 2-Gm. sample; 250 mg. per oz., 4-Gm. sample. An accurately weighed sample of suitable size was placed in a Waring Blendor, 100 ml. of a solution of equal parts of acetone and 0.1 *M* aqueous hydrochloric acid was added, and the mixture was blended for five minutes. The contents of the Blendor were poured into a 150-ml. beaker, covered with a watch glass, and allowed to cool to room temperature (about twenty minutes). Ten milliliters of the supernatant liquid was pipetted into each of two G. S. Erlenmeyer flasks. The solution pipetted does not have to be clear. To one flask was added 5.0 ml. of a solution of four parts of the acetone-hydrochloric acid used previously and one part of 0.4 *M* sodium hydroxide. To the other flask was added 5.0 ml. of a standard solution prepared as for Group B. Each solution (*p*H about 2.9) was placed in the polarographic cell, purged with nitrogen for five minutes, and polarographed over the potential range 0 to −1 v. The diffusion currents were calculated in microamperes and the appropriate values were substituted in the following formula:

$$\text{Mg. of riboflavin per oz.} = \frac{i_a\ (\mu a)}{i_b - i_a\ (\mu a)} \times \frac{\text{mg. riboflavin (U.S.P.)/50 ml.} \times 28.35}{\text{wt. of sample in Gm.}}$$

where i_a is the diffusion current found for the sample and i_b is the diffusion current for the sample plus standard. The results obtained by this procedure are compared in Table III with those obtained by the fluorometric assay for a representative group of vitamin mixtures.

TABLE III.—A COMPARISON OF THE POLAROGRAPHIC AND FLUOROMETRIC ASSAY METHODS FOR RIBOFLAVIN IN GROUP C MATERIALS

Designation	Diluents	Polarographic Assay, Mg./Oz.	Fluorometric Assay, Mg./Oz.
A	Cornstarch[a]	1,035; 1,073	1,160
B	Cornstarch[a]	260; 265	262
C	Cornstarch	1,088	1,080
		1,078; 1,095	1,100
		1,115; 1,150	1,100
D	CDDG[b]	279; 284	273
E	CDDG[b]	281; 283	299
F	Corn meal	444; 462	452
		477; 483	469
		457; 458	430
G	CDDG[b,c]	138; 141	134

a Also contains calcium pantothenate.
b CDDG is corn distiller's dried grains.
c Also contains calcium pantothenate, niacin, and choline chloride.

DISCUSSION

The fluorometric assay method is usually considered to have a precision of about ±2 relative per cent whereas the polarographic assay has a precision of about ±3 relative per cent. Thus the agreement between the results obtained by the fluorometric assay method and by the polarographic method is within the precision of the respective methods. The polarographic method is most useful for the assay of riboflavin in mixtures, since in this case the pretreatment before the instrumental determination is much less than for the fluorometric method. The polarographic method is a reliable alternate assay method for riboflavin and its use by laboratories equipped for such determinations is urged.

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Studies on Antacids. IV. Adsorption Effects of Various Aluminum Antacids upon Simultaneously Administered Anticholinergetic Drugs*

By I. W. GROTE and MARIBELLE WOODS†

A study was made of the possible adsorption of various drugs often given simultaneously with aluminum compounds in the treatment of gastric hyperacidity. The approximate LD_{90} for each drug upon oral administration to mice was found, and the same dose was then given in admixture with various aluminum preparations to determine if the same LD was obtained. A number of drugs tested were adsorbed by aluminum hydroxide gel, while certain other aluminum antacids did not cause adsorption to the same extent, if at all.

THE ADSORPTION effect of various aluminum compounds upon organic and inorganic compounds has long been known. The use of Lloyd's reagent (sodium aluminum silicate) for such purposes dates back some seventy-five years. Much of the work in the field of chromatographic adsorption has made use of various preparations of aluminum oxide. It has been shown (1) that aluminum hydroxide gel readily adsorbs up to 90% of the total activity of aureomycin and terramycin in a nonrecoverable form. Recently Seifter, *et al.* (2), have shown that aluminum hydroxide gel and hydrated alumina powder change the effect of various anticholinergetic drugs when tested in rats by a lacriminatory blocking action. Since a number of anticholinergetic drugs are often given along with the aluminum antacids for their synergistic effect in the control of ulcer pain, the authors have investigated the possible adsorption of several of these drugs when administered together with the antacid.

METHOD

The method of test used in the present investigation consisted essentially of finding the approximate mouse oral LD_{90} dose for the drug to be tested, and then giving this same dose of drug in admixture with the aluminum preparation to determine if the same LD effect was obtained after admixture as before. If the drug was adsorbed by the aluminum preparation in a form either nonrecoverable or recoverable only very slowly in the intestinal tract of the mouse, the toxicity of the compound simultaneously administered should be decreased. Presumably this would indicate whether the desired synergistic effect in the human patient would also be affected when the two drugs are administered together.

As Seifter, *et al.* (2), have pointed out in an article published after the present investigation was begun, such a study does not permit the use of ratios of aluminum antacids to synergistic anticholinergetic drugs in ratios which even approximate those actually used in human therapy. In the case of some of the atropine-like drugs, the actual LD_{90} in milligrams for mice is about the same as that for humans. This factor, however, should merely minimize the effect which would be found if the actual therapeutic ratios of drug to antacid were used, since the aluminum compound would probably show a still greater adsorption if used in a much higher ratio.

The maximum dose of the magma of the aluminum compound that can conveniently be given orally to a mouse by stomach tube is 200 mg. of the aluminum antacid suspended in a volume of 1 cc. This introduces another undesirable factor in that the huge dose of antacid completely neutralizes the stomach acidity and the drug-antacid mixture passes through the intestinal tract in a viscous mass. The lack of acidity in the stomach may not be important from the standpoint of absorption, as Travell (3) has shown that alkaloids are more readily absorbed from a nonacid stomach than from one of normal acidity. The relatively huge mass of antacid in the mouse intestine compared to that which would actually be used in human therapy, however, shows that the results obtained in this work are of a qualitative nature only. However, the fact that some drugs demonstrate unchanged toxicity when given in admixture with the antacid shows that the method is valid in principle.

The antacids tested consisted of a highly active dried alumina gel, U. S. P., dihydroxy aluminum aminoacetate, N. N. R. (powder), two commercially available aluminum hydroxide magmas, and one commercially available aluminum phosphate magma. For the dried aluminum compounds, suspensions were prepared containing 200 mg. of antacid plus the desired amount of anticholinergetic drug for each 1-cc. dose. Since the commercially available magmas contain considerably less than 200 mg. of dried aluminum compound per cc., it would be expected that the magma test suspensions would be less adsorptive than the dried aluminum compound. Because the dry compounds are used in medicine in approximately equal doses by weight, a direct comparison by weight is justified.

* Received August 22, 1952, from the Brayten Pharmaceutical Co., Chattanooga, Tenn.
Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.

† Thanks are due to Dr. John C. Krantz, Jr., for suggesting that adsorption of drug by antacid could be most easily shown by reduction of an LD_{90} mortality obtained with the drug alone.

TABLE I.—ACUTE ORAL TOXICITY OF DRUGS BOTH FRESHLY MADE UP AND AFTER STANDING FOUR DAYS IN MIXTURE WITH VARIOUS ALUMINUM ANTACIDS

Drug	Dose Mg.[a]	Drug Only	Dihydroxy Aluminum Aminoacetate, 200 Mg.		$Al(OH)_3$ Gel, Dried, 200 Mg.		Commercial Magmas		
			Fresh	4-Day	Fresh	4-Day	$Al(OH)_3$ Magma A, 1 Cc., 4-Day	$Al(OH)_3$ Magma B, 1 Cc., 4-Day	$AlPO_4$ Magma, 1 Cc., 4-Day
Atropine sulfate	30	5/5[b] 100%	5/5 100%	...	5/5 100%	...	...	...	...
	20	29/30 97%	29/30 97%	24/30 80%	23/30 77%	3/30 10%	26/30 87%	...	25/30 83%
Homatropine hydrobromide	30	5/5 100%	5/5 100%	...	5/5 100%	...	...	...	...
	20	28/30 93%	26/30 87%	23/30 77%	17/30 57%	0/30 0%	12/30 40%	19/30 63%	22/30 73%
Homatropine methyl bromide	50	5/5 100%	...	...	...	...	...	...	...
	40	22/25 88%	23/25 92%	18/25 72%	22/25 88%	19/25 76%	13/25 52%	23/25 92%	24/25 96%
Mixed belladonna alkaloids as sulfates	20	5/5 100%	...	...	5/5 100%	...	...	...	...
	15	27/30 90%	20/30 67%	25/30 83%	17/30 57%	5/30 17%	8/20 40%	...	4/20 20%

[a] Since all mice weighed within ±2 Gm., they received the same amount of drug and the same amount of antacid.
[b] Mortality is expressed as the ratio of the number of female mice dead in twenty-four hours to the number of mice tested.

In order to test the effect of time upon the adsorption of the drug by the antacid, tests were made upon antacid-drug mixtures both prepared immediately before oral administration to the mouse and those in which the drug-antacid magmas were mixed and allowed to stand for four days before administration. Mice of the same strain were used throughout the study, and variations of weight in individuals used in a single test series were held to ±2 Gm. This permitted the use of a single weight of drug-antacid within each series, rather than one based on a mg./Kg. dose. The mixture to be tested was given orally, using a tuberculin syringe fitted with a blunt No. 18 needle. Animals were then observed for twenty-four hours and the deaths which occurred during this period were recorded. From 20 to 30 mice were used for each dosage level. The results are given in Table I.

Examination of the data indicates that a freshly prepared magma made from an "active" aluminum hydroxide U. S. P. shows some adsorption of atropine sulfate, homatropine hydrobromide, and mixed belladonna alkaloids as sulfates, even when given immediately after mixing. After the magma has stood for four or five days, adsorption as measured by toxicity tests is nearly complete. Adsorption is less in the case of the commercially prepared aluminum hydroxide magmas after four days' standing but is still marked, as the toxicity in some cases is reduced to about half that found with the alkaloid alone. Commercial aluminum phosphate magma does not show a marked adsorption effect except in the case of the mixed belladonna alkaloid sulfates, where the mortality was reduced from 90% using the alkaloid to 20% after four days' standing in the presence of the magma. Dihydroxy aluminum aminoacetate N. N. R. shows markedly less adsorption; the maximum change observed in the case of homatropine hydrobromide is from an LD_{96} to an LD_{77} in the same dosage after four days' standing in the magma.

Homatropine methyl bromide is adsorbed to a lesser degree by all of the antacid drugs tested; the maximum adsorption shown is a reduction of an LD_{88} to an LD_{52} in the case of one commercial aluminum hydroxide magma after four days' standing.

In conclusion, aluminum hydroxide magmas show marked adsorption effects as measured by reduction in acute toxicity in mice with atropine sulfate, homatropine hydrobromide, and belladonna alkaloids as sulfates and have little or no effect on the toxicity of homatropine methyl bromide. Dihydroxy aluminum aminoacetate shows much less adsorption of the various alkaloids as measured by the toxicity. Except for belladonna alkaloids where marked adsorption is noted, commercial aluminum phosphate magma shows only little adsorptive effect on the alkaloids tested.

The number of actual tests reported are relatively few in number; however, it was the purpose of the authors to report on the procedure rather than to cover the whole field of aluminum adsorption. With this simple procedure, therapeutic effectiveness of mixtures can be more accurately predicted before clinical study is begun.

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The Synthesis of Some Simple *n*-Alkyl Esters of 4-Aminohexane-1-carboxylic Acid*,†

By R. K. PATEL and OLE GISVOLD‡

Four simple (ethyl through *n*-amyl) esters of *cis*-4-aminocyclohexane-1-carboxylic acid have been prepared for testing for their anesthetic activities.

THE EXISTENCE of so many structurally diversified organic compounds that possess local anesthetic activity complicates the attempt to explain the mode of action of this class of compounds on the basis of competitive inhibition or the interference with normal functioning of a simple limiting factor. However, this does not preclude the development of fundamental information about any one class of these compounds. It was for this purpose that the compounds reported in the present paper were prepared.

Some of the esters of *p*-aminobenzoic acid have been established as very useful local anesthetics, e. g., both ethyl- and butyl *p*-aminobenzoates. The complete reduction of the benzene ring in these compounds would destroy their aromatic character, presenting an opportunity to compare the contribution of the benzene ring to anesthetic activity with that of cyclohexane. Since the reduction of *p*-aminobenzoic acid yields a *cis*- and a *trans*-hexahydro-*p*-aminobenzoic acid, respectively, two series of esters were available for comparison. Only a few *cis*- esters are reported in the present paper.

It is also known that hexahydro-*p*-aminobenzoic acid can antagonize sulfanilamide (1). This antagonism is believed to be due to the biological dehydrogenation of the hexahydrocompound to yield the active *p*-aminobenzoic acid. Apparently it has not been shown whether or not the hexahydro-*p*-aminobenzoic acid esters undergo biological dehydrogenation. Such information would be necessary in order to establish whether any local anesthetic activity obtained from the reduced esters resulted from the esters themselves or from their biologically dehydrogenated products.

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† Abstracted from a thesis submitted to the Graduate Faculty of the University of Minnesota by Ramanbhai K. Patel in partial fulfillment of the requirements for the degree of Master of Science.
‡ Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota.

EXPERIMENTAL

The esters were prepared by condensing hexahydro-*p*-amino-benzoyl chloride with the respective alcohols.

Preparation of 4-Aminocyclohexane-1-carboxylic Acid.—*Para*-aminobenzoic acid was reduced at room temperature and low pressure in an aqueous medium with platinum oxide as the catalyst according to Wendt's procedure (2). The mixture of *cis-trans* forms of 4-aminocyclohexane-1-carboxylic acid were also resolved by the procedure with some modifications.

Preparation of the Acid Chloride of 4-Aminocyclohexane-1-carboxylic Acid.—Two grams of *cis*-4-aminocyclohexane-1-carboxylic acid were gently refluxed on a steam bath with an excess of thionyl chloride. The excess thionyl chloride was removed under vacuum and the crude acid chloride hydrochloride obtained was used without further purification.

Preparation of the Simple Alkyl Esters of Cis-4-aminocyclohexane-1-carboxylic Acid.—These esters were prepared by treating the appropriate anhydrous alcohol with the previously described crude acid chloride. The reaction was exothermic and accompanied by a loss of hydrogen chloride gas. The resulting ester was recrystallized from the alcohol used in the preparation of the ester.

Table I summarizes the results obtained in the synthesis of the simple alkyl esters.

TABLE I.—ESTERS OF CIS-4-AMINOCYCLOHEXANE-1-CARBOXYLIC ACID

No.	Ester Residue	M. P., °C.	Analysis, % Calcd.	Found
1	Ethyl	193–194	C, 52.2 H, 8.7	C, 52.2 H, 8.75
2	*n*-Propyl	184–185	C, 54.3 H, 9.05	C, 54.5 H, 9.09
3	*n*-Butyl	174–175	C, 56.2 H, 9.37	C, 56.1 H, 9.41
4	*n*-Amyl	169–170	C, 57.9 H, 9.73	C, 58.2 H, 9.88

Anesthetic Activity.—A preliminary test indicated that these compounds possess anesthetic activity. More complete testing is required.

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Notes

A Note on the Effect of Nembutal (Pentobarbital Sodium) and Ether on the Thromboplastic Potency of Mouse Brain*, †

By D. C. SCHECHTER and J. W. E. HARRISSON

DURING the course of studies on barbiturate poisoning, the authors became interested in its influence on the mechanism of blood coagulation. Seegers (1) has recently reviewed the effect of various anesthetics on the clotting of blood, with particular reference to postoperative thromboembolic episodes. Aykut (2), Levy and Conroy (3), and Fitzgerald and Webster (4) have described shortening of the prothrombin and coagulation times by Nembutal. Mendenhall (5) and Searls and Essex (6) observed that ether anesthesia lowers the clotting time of whole blood. Levy and Conroy (3) demonstrated an increased prothrombin concentration in the blood of patients under deep ether anesthesia. Borgström (7) reported a drop in prothrombin index after combined nitrous oxide-ether anesthesia.

The authors have been interested in the effect of barbiturates and ether on the thromboplastic activity of the brain.

METHODS

A total of 102 male Swiss mice of 15–25 Gm. body weight were used for this study, and were divided equally among the various groups. The brains of each group were stripped free of meninges and adhering clots, pooled, and tested as a group. The method of Gollub, Kaplan, and Meranze (8) was used for the preparation of pooled brain matter and the extraction of the thromboplastic substance. Dilutions of the "100%" thromboplastin (i. e., 0.5 Gm. of desiccated tissue to 10 ml. of physiological saline) were made with 1:1 dilution of a solution containing 0.85% NaCl:0.025 *M* $CaCl_2$ mixture. A modification of the Quick One-stage procedure for determinations of the prothrombin time was employed (6). Homologous plasma from unanesthetized Swiss mice was used in these tests.

RESULTS

Table I demonstrates the difference in the thromboplastic potency of brain suspensions from mice killed with 168 mg./Kg. of Nembutal or inhalation of ether vapors and those from animals sacrificed by decapitation. It can be noted from this table that reduction of the clotting time is consistently more pronounced in the Nembutal group than in the ether group. This reduction is also more evident in the lower concentrations of thromboplastin.

TABLE I

Concn. Thromboplastic Suspension, %	Prothrombin Time, Sec.		
	Control Group	Nembutal Group	Ether Group
100	7.3	7.3	8.5
50	12.7	7.7	9.6
25	17.1	9.3	11.7
10	23.5	12.3	15.2
1	53.6	22.6	26.8

Gollub, *et al.* (9), have demonstrated a qualitative variation in thromboplastic action between the different morphological parts of the brain, particularly between the medulla and the rest of the brain. In view of this observation, it seemed worth-while to investigate the effect of the drugs on these separate parts of the brain.

Table II sets forth the results which were obtained. It appears that the tissue preparations from the Nembutal series are less potent than those from the ether group, this being more manifest in the lower thromboplastin dilutions and, especially, with the medulla. It is possible that in the brain *in toto* a balance of clotting activators and inhibitors is responsible for this discrepancy.

TABLE II

Concn. Thromboplastic Suspension, %	Prothrombin Time, Sec.			
	Nembutal Group		Ether Group	
	Brain Minus Medulla	Medulla	Brain Minus Medulla	Medulla
100	7.9	10.4	6.9	7.7
50	9.2	12.6	9.3	9.5
25	10.8	15.2	10.4	12.2
10	14.5	19.3	12.2	14.6
1	25.8	...	24.0	29.7

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* Received August 22, 1952, from the LaWall Memorial Laboratory of Pharmacology and Biochemistry, Philadelphia College of Pharmacy and Science, Philadelphia, Pa.
Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.
† This project was supported in part by a grant from Eli Lilly & Co.

A Note on the Determination of Isonicotinic Hydrazide by a Volumetric Method*

By HANS HARTING

THE DETERMINATION of acid hydrazides has become of general importance since the introduction of isonicotinic hydrazide in the treatment of tuberculosis. The known analytical methods leave much to be desired with respect to application in most clinical laboratories, due to the difficulty of execution as well as to the rather complicated installations required.

The method presented here has been found to have a very high degree of precision and the equipment needed is extremely simple.

Acid hydrazides, hydrazine itself, as well as its salts, are easily oxidized, quantitatively transforming the hydrazine-nitrogen into nitrogen gas under certain conditions. Hydrazine itself reacts in the following manner:

$$NH_2NH_2 + O_2 \rightarrow N_2 + 2H_2O$$

In a similar reaction, acid hydrazides are oxidized, the final result depending on the amount of oxidizer used. With insufficient oxidation, acylated hydrazones are formed:

$$2RCONHNH_2 + O \rightarrow RCH{=}NNHCOR + H_2O + N$$

With sufficient oxidizer, however, acid hydrazides are decomposed, forming the corresponding aldehydes, and at the same time all the hydrazide-nitrogen is evolved in the form of nitrogen gas according to the equation:

$$RCONHNH_2 + O \rightarrow RCHO + H_2O + N_2$$

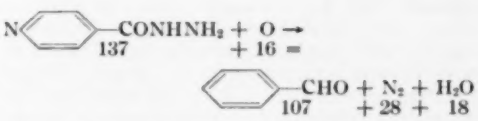
Using surplus oxidizer, the aldehyde will be further oxidized to the corresponding acid, leaving, of course, the nitrogen gas unaltered. In the oxidation of acid hydrazides with surplus oxidizing agent, all of the hydrazide-nitrogen will be evolved as nitrogen; in other words, the amount of nitrogen formed in the oxidation of acid hydrazides indicates stoichiometrically the quantity of acid hydrazide used.

As oxidizing agents, potassium permanganate, potassium dichromate, or, preferably, potassium ferricyanide can be employed. The latter was found to be by far the most convenient oxidizer when used in the presence of potassium hydroxide.

The reaction velocity is so great that after a few minutes at room temperature all the nitrogen is evolved.

EXPERIMENTAL

The execution of the analysis is extremely simple: an aqueous solution of acid hydrazide, i. e., isonicotinic hydrazide, is mixed with a solution of potassium hydroxide, and an excess of potassium ferricyanide solution is added. The nitrogen gas evolved is measured, and its weight is calculated in accordance with temperature and pressure. The amount of isonicotinic hydrazide is calculated as follows:



One milligram of nitrogen gas represents 137/28 mg. of isonicotinic hydrazide; thus the quantity of nitrogen multiplied by 4.893 corresponds to the amount in milligrams of isonicotinic hydrazide used.

Since only hydrazine compounds form gases when mixed with alkaline ferricyanide, this method can be employed with the same accuracy in the analysis of isonicotinic hydrazide in mixtures with other substances, i.e., starch, urea, etc. This permits the drug content of tablets to be analyzed directly after disintegration in water. Furthermore, in clinical analysis the biological liquids (sputum, urine, pleura exudate, cerebrospinal liquid, etc.) can be used without extraction, purification, or other inconvenient operations. It shall be noted, however, that amithiozone (*p*-acetylaminobenzaldehydethiosemicarbazone) will also be decomposed by alkaline ferricyanide forming nitrogen. The method thus cannot be employed in the presence of this drug.

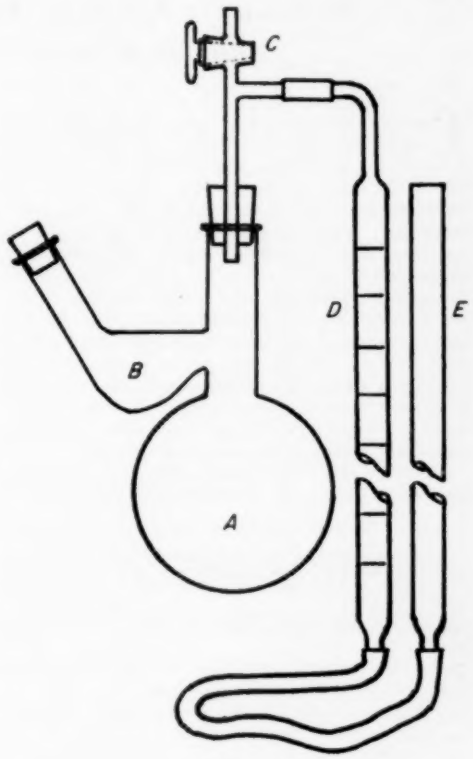


Figure 1

* Received September 9, 1952, from Harting y Cia. Ltda., Santiago de Chile.

The apparatus (Fig. 1) used most advantageously for the determination of isonicotinic hydrazide (hydrazine itself or other acid hydrazides) consists of a special flask which prevents previous mixing of the ingredients. Bulb A has a capacity of 80 ml., and that of side-bulb B is 20 ml. After filling the gas burette D (an ordinary measuring pipette of 20-ml. capacity) with distilled water to the zero mark by means of the small leveling bulb E (or measuring pipette), bulb A is charged with the aqueous solution of isonicotinic hydrazide (i. e., one 50-mg. tablet of the drug disintegrated in 20 ml. of water) and 10 ml. of 20% potassium hydroxide solution is added. Then the flask is closed with a stopper carrying a T-tube with stopcock C. Last, 10 ml. of a 20% solution of potassium ferricyanide is introduced into bulb B by pipette, avoiding previous mixing with the contents of bulb A, and bulb B is closed with a stopper. After standing for about ten minutes, the stopcock is closed and the liquid in bulb B is transferred to bulb A by inclining the support on which the whole apparatus is fixed. The apparatus is shaken gently for two minutes (avoiding direct contact with the hands or heat radiation) and left standing for fifteen minutes. The nitrogen evolved displaces an equal quantity of air in the system which is measured in the burette. From the amount of moist air measured, the quantity of nitrogen is calculated, using the ordinary nitrogen tables. By multiplying the amount of nitrogen in milligrams by 4.893, the amount of isonicotinic hydrazide is found.

Under the conditions described, 50 mg. of isonicotinic hydrazide evolved 9.11 ml. of nitrogen at 12° and 716 mm. pressure. Found: 50.34 mg. of isonicotinic hydrazide.

SUMMARY

A method has been described for the quantitative determination of isonicotinic hydrazide by oxidation in alkaline solution with ferricyanide. The hydrazide-nitrogen is transformed quantitatively into nitrogen gas, which is then measured.

The analytical method can be carried out in presence of any foreign matter, since only hydrazine compounds and derivatives of thiosemicarbazide form gases under given conditions. Its application in clinical laboratories was indicated.

An apparatus for carrying out the analysis has been described. Investigation of this subject is being continued.

A Preliminary Note on a Pharmacological Investigation of the Hypotensive Action of *Rauwolfia serpentina* Benth.*

By JOHN W. NELSON† and CARL A. SCHLAGEL‡

THIS INVESTIGATION was begun during the summer of 1951 when the hypotensive potentialities of *Rauwolfia serpentina* were brought to the attention of the senior author by representatives of the Columbus Pharmacal Company of Columbus, Ohio. Preliminary sighting experiments were begun at that time, and after confirming that *Rauwolfia* did possess hypotensive activity, a complete survey of the scientific literature was made. Various methods of extraction and isolation of the active constituents were used and pharmacological and toxicity studies made. On the basis of the results obtained from these studies arrangements were made in May, 1952, for a research grant from the Columbus Pharmacal Company to carry out a complete investigation of the hypotensive action of *Rauwolfia*.

Results of this investigation show that up to this time preparations of *Rauwolfia serpentina* Benth. are relatively potent hypotensive agents as indicated in anesthetized normal dogs. Comparison tests with other known hypotensive agents indicate a potency of about one-tenth that of Veratrum Viride in terms of crude drug. Of two known varieties and four unknown varieties obtained from several different sources, the Dehra Dun variety shows the greatest activity. The hypotensive activity resides in the alkaloidal fraction. Testing of the separated alkaloids shows serpentine to be decidedly more potent than the ajmaline alkaloids. Both the alcohol extract and the serpentine alkaloid produce a fall in carotid blood pressure when administered intragastrically as well as intravenously. The reduction in blood pressure is gradual and sustained (in the case of intragastric administration for more than four hours).

No untoward side effects have been observed with either the crude alkaloidal extract or the serpentine alkaloid and toxicity studies indicate a high therapeutic index.

Further pharmacological and clinical studies are in progress and the completed results will be published at a later date.

* Received April 12, 1953, from The Ohio State University College of Pharmacy, Columbus, Ohio.

† Professor of Pharmacy, The Ohio State University College of Pharmacy.

‡ Columbus Pharmacal Company Fellow, The Ohio State University College of Pharmacy.

Book Notices

Antibiotics—A Survey of Their Properties and Uses. Published by direction of the Council of the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London, 1952. xii + 290 pp. Illustrated. 23 x 15 cm. Price 25*s* (Postage 10*d*).

The first edition of this book, which was published in 1946 under the title "Penicillin: Its Properties, Uses and Preparations," was reviewed in THIS JOURNAL, **36,** 96(1947). This edition has been rewritten to include streptomycin, chloramphenicol, aureomycin, and terramycin, which are now in general use in Great Britain. The bacterial antibiotics, tyrothrycin, bacitracin, and the polymyxins are included in or appended to the general discussions. It is pointed out that as the book went to press (May, 1952) penicillin, streptomycin, chloramphenicol, and aureomycin were the only antibiotics available in more than experimental quantities in the United Kingdom, and that terramycin had become available for the treatment of a small number of diseases.

The tests and standards given in the text do not include those that are used and recognized in the United States. Although the book is written for the particular consideration of the pharmacists and physicians of the United Kingdom, there is much sound information concisely presented and well documented which will appeal to everyone who is interested in this expanding field.

The properties and uses of the antibiotics are discussed under the following headings: commercial manufacture, chemistry, stability, standards and methods of assay, experimental background, clinical applications, veterinary practice, and pharmacy.

Statistical Method in Biological Assay. By D. J. FINNEY. Hafner Publishing Co., New York, 1952. Illustrated. xix + 661 pp. 22 x 15 cm. Price $11.

A comprehensive account of designs and statistical analyses for biological assays is presented in this book by Dr. Finney, who lectures on the Design and Analysis of Scientific Experiment at the University of Oxford. Although the author has assumed that his readers are reasonably familiar with the basic statistical techniques used in research, he gives detailed explanations and illustrations of problems relative to the statistics of assays.

Dr. Finney emphasizes the role of the professional statistician, and he states that the statistician must be prepared to advise on the plan and economy of each investigation with regard to its operational efficiency for a particular purpose. The cycle of design, analysis of data, report, and integration is shown to recur frequently in biological assay. The fact that Dr. Finney has been and is one of the foremost practitioners in the field of collaborative statistical practice in the development and application of bioassay techniques makes this an authoritative reference volume.

Neomycin. By SELMAN A. WAKSMAN. Rutgers University Press, New Brunswick, N. J., 1953. xiv + 219 pp. 16 x 23.5 cm. Price $4.

Neomycin is an antibiotic substance produced by a strain of *Streptomyces fradiae.* The substance was first isolated by Dr. Waksman and his associates of the laboratories of the Department of Microbiology of the New Jersey Agricultural Experiment Station, Rutgers University. Its isolation and partial characterization were the result of an effort to obtain an antibiotic that would possess all of the desirable qualities of streptomycin, but be free from some of its toxic manifestations. Early investigations indicated that neomycin might be such an agent. It is water soluble and acts upon a great variety of bacteria. Investigation has revealed that this new antibiotic is also characterized by certain potential toxicity and allows some development of resistance. Despite this, it was considered to possess sufficient desirable properties to justify its detailed study. The results of this study are outlined in *Neomycin* in which all phases are adequately covered.

The book is divided into 14 chapters in which the assay and standardization of neomycin, neomycin-producing cultures, nutrition of *S. fradiae,* neomycin production, isolation, and its physical and chemical properties, are discussed as well as its bacteriostatic and bactericidal properties, clinical effects, and its chemotherapeutic potentialities.

Although neomycin was first isolated in 1949, the literature relating to it is quite extensive. The book is documented throughout with references to the 224 reports that had appeared at the time the book went to press. This latest book by Dr. Waksman and his associates will appeal most strongly to specialists in the field of antibiotics, but it also makes interesting reading for anyone wishing to obtain background information concerning the methods employed in the screening of cultures isolated from soils and other sources, and the isolation, characterization, and determination of activity of an antibiotic substance.

Accepted Dental Remedies 1953. 18th ed. Council on Dental Therapeutics, American Dental Association, Chicago, Ill., 1952. xvi + 208 pp. 20.5 x 16 cm.

Accepted Dental Remedies is published annually under the supervision of the Council on Dental Therapeutics of the American Dental Association. The book is designed to include listings of currently accepted commercial products and a description of all official and unofficial therapeutic items which are of demonstrated usefulness in dental practice. Because of its comprehensive nature, it is of especial in-

terest to practicing pharmacists who require information concerning the drugs used in dental practice.

In addition to information relating to drugs used by dentists, *Accepted Dental Remedies* also includes chapters on the provisions for the acceptance of products by the Council on Dental Therapeutics, dental formulas and tables useful in general dental practice, and on symptoms and treatment of acute poisoning. The book is provided with an index to distributors and an unusually well-designed general index. For pharmacists who are interested in supplying dentists with the preparations they use in the office, the chapter on formulas and tables is particularly significant.

Glycerol. Edited by CARL S. MINER and N. N. DALTON. American Chemical Society Monograph Series. Reinhold Publishing Corp., New York, 1953. xiv + 460 pp. 16 x 23.5 cm. Illustrated. Price $12.

This book is the result of a collaborative effort involving leading research scientists and is edited under the sponsorship of the Glycerine Producer's Association of America. This new addition to the American Chemical Society's Monograph Series furnishes complete and up to date information on the sources, recovery, refining, and uses of glycerin. All of the recent important developments in the field are fully discussed. The treatment of the chapters dealing with synthetic glycerols, purification by ion-exchange, standards and specifications, the physiological action of glycerin and its uses and application are of particular interest to pharmacists and pharmaceutical chemists. Glycerin plays an unusually important role in the manufacture of liquid pharmaceutical preparations and no satisfactory substitute has been found. Because of the pharmaceutical importance of glycerin, this book should be of special interest to all members of the pharmaceutical profession.

The book is completely documented with references to original publications dealing with all phases of glycerin production and applications.

The printing and binding are of the type that has characterizeo the American Chemical Society's Series of Chemical Monographs and the book is provided with a complete and adequate index. *Glycerol* can be recommended highly as a source of information on any problem involving glycerin.

Scientific Terminology. By JOHN N. HOUGH. Rinehart and Co., Inc., New York, 1953. xiii + 231 pp. 13.5 x 21 cm. Price $3.50.

This book, according to the author, who is a Professor of Classics at the University of Colorado, is the outgrowth of many years' teaching of students pursuing scientific curriculums. It is designed to combine into a single volume premedical and biological terms and nomenclature of Latin and Greek origin. The book is divided into thirteen chapters including the following headings; The Greek and Latin Alphabets; Formation of English Words: Roots, Stems, Combining Forms; Formation of English Words: Suffixes; Formation of English Words: Prefixes; General Scientific Vocabularies; Latin Forms and Latin Grammar in Scientific Nomenclature; Medical and Pathological Terminology; Bacteriological Nomenclature; Pharmaceutical Terminology; Biological Terms; The Linnaean System of Biological Nomenclature; and the Reading and Writing of Latin Descriptions.

The chapter on "Pharmaceutical Terminology" presents the essentials of Latin nomenclature required in present-day pharmaceutical practice. The chapter on "The Linnaean System of Biological Nomenclature" serves a useful purpose in presenting the most important parts of the system.

Calendar of the Pharmaceutical Society of Great Britain 1952–1953. The Pharmaceutical Society of Great Britain, 17 Bloomsbury Square London, W.C. 1, England, 1953. 305 pp. 14.5 x 22 cm. Price 12*s.* 6*d.*

This valuable book was reviewed somewhat in detail [THIS JOURNAL **51,** 54(1952)]. The new edition follows the same design as the one which appeared in 1951. This *Calendar* has been revised where necessary and will continue to serve its intended function of keeping pharmacists of Great Britain informed on matters related to the Pharmaceutical Society, its activities, its educational requirements, and the legal information with which pharmacists are required to be familiar.

Standard Values in Blood. ERRETT C. ALBRITTON, Editor. Prepared under the direction of the Committee on the Handbook of Biological Data, American Institute of Biological Sciences, the National Research Council. W. B. Saunders Co., West Washington Square, Philadelphia 5, Pa., 1952. x + 199 pp. 21.5 x 28 cm. Price $4.50.

This compilation includes the more basic established data on the composition and reactions of blood, of interest to scientists working in the various fields of biology, including the medical sciences. The compilation was made as the result of a contract between the National Research Council and the Wright Air Development Center and the actual work was done by the Committee on Handbook of Biological Data. The scope of the book and the subject of the tables can best be indicated by listing the several chapter titles. These are: Physical Properties; Coagulation Phenomena; Blood Groups; Erythrocytes and Hemoglobin; Leukocytes; Blood and Bone Marrow Cells; Water, Carbohydrate, Lipid, Protein; Amino Acids, Non-Protein Nitrogen; Phosphorus, Sulfur; Vitamins, Hormones, Enzymes; Electrolytes, Minerals, Gases, Acid-Base; Effects of Radiation, Storage; and Therapeutic Agents.

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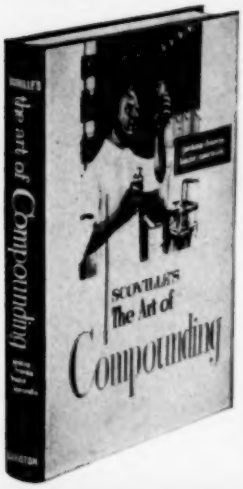

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